Neuropeptidergic regulation of digestion and food-dependent reproduction in the adult female flesh fly, *Sarcophaga crassipalpis*

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Abstract

Many flesh fly species are anautogenous. Due to their life style, these flies have a negative impact on livestock since they can cause myiasis.

Anautogeny is a kind of reproductive strategy, where adult female insects require a proteinaceous meal in order to initiate vitellogenesis - the large scale synthesis of yolk proteins and their subsequent uptake by the developing oocytes. The expression of yolk protein precursor genes is tightly controlled by the nutritional state of the organism. It remains arrested at the previtellogenic stage, until the protein meal derived signal(s) activates particular signaling pathways.

The protein meal intake induces an increase in midgut proteolytic activity, which is mainly assured by trypsin and chymotrypsin, and subsequently the production of yolk proteins starts. Digestion, as all other physiological processes, is very complex and regulated by a multitude of external and internal stimuli, such as food perception or release of neuropeptides from either the gut or brain. Time series decapitations and rescue experiments of liver-fed flies, indicated the need of a peptide factor to be released by *corpora cardiaca* within 4 hours post protein feeding, in order to assure complete protein meal digestion. After this critical period of 4 hours, manipulation did no longer affect proper proteolysis.

Quantitative differential peptidomic analysis of *corpora cardiaca*, dissected respectively from sugar and liver (5 hours post protein feeding) fed flies, identified a unique and consistent decrease in the stored amount of adipokinetic hormone. Moreover, injection of this neuropeptide into liver-fed decapitated flies and into sugar-fed intact flies resulted in a dose-dependent, enhanced midgut proteolytic activity, up to the level observed in intact protein-fed flies. None of the other peptides that were present in the examined tissue was able to generate similar, long-term effects in sugar-fed intact flies, nor in decapitated liver-fed insects. This strongly suggests a role of adipokinetic hormone in digestion regulation and consequently food-dependent reproduction. This peptide functions via activation of the newly identified adipokinetic hormone receptor that belongs to the family of G protein-

coupled receptors. In line with the main function of this adipokinetic hormone being involved in energy mobilization, the receptor was found to be most abundantly expressed in the fat body, a known energy storing organ. Nonetheless, the adipokinetic hormone receptor is also highly expressed in the brain, foregut and hindgut. Interestingly, after protein feeding the receptor transcript number was reduced in almost all tissues. These changes most probably enforce the use of ingested energy carrying molecules prior to stored energy mobilization. Pharmacological characterization indicated that the receptor can be activated by two related dipteran adipokinetic hormone ligands, with an EC_{50} value in the low nanomolar range.

Digestive activity and subsequent ovarian development could be pharmacologically manipulated. Abdominal injection of 6-hydroxydopamine, dose-dependently, prohibited the increase of the midgut proteolytic activity in liver-fed flies and as such caused a similar effect as fly decapitation. In *S.crassipalpis* this inhibition of the liver feeding elicited increase in protein digestion is seemingly caused at post-transcriptional level. In flesh flies, this irreversible chemical decapitation, by interrupting the brain-gut dopaminergic signaling, could be used as a research tool for the controlled inhibition of the midgut proteolytic activity and subsequent ovarian development. Inhibition of the ovarian development is most probably indirect, due to a deficiency in free circulating amino acids which are needed for yolk protein synthesis.

Abstract (Nederlands)

Vleesvliegen zijn anautogene insecten. Door hun levensstijl hebben deze vliegen een negatieve impact voor mens en dier daar zij myiasis kunnen veroorzaken.

Anautogenie is een vorm van voortplantingssysteem waarbij de volwassen wijfjes van deze insecten een proteïnerijke maaltijd nodig hebben om de grootschalige synthese van de dooiereiwitten of vitellogenines in te leiden. De expressie van de precursoren van deze dooiereiwitten wordt strikt gereguleerd door de voedingstoestand van het organisme. Deze blijft op het previtellogene niveau tot wanneer een proteïnemaaltijd de signaaltransductiecascade activeert.

De proteïne inname veroorzaakt een toename van de enzymatische activiteit in de middendarm, die vooral verzekerd wordt door enzymen als trypsine en chymotrypsine, en vervolgens start de vitellogeninenproductie. De spijsvertering, zoals andere fysiologische processen, is zeer complex en wordt gereguleerd door een veelheid aan externe en interne prikkels zoals voedselwaarneming of de vrijgave van neuropeptiden uit de darm of de hersenen. Tijdsreeksen van decapitatie en rescue experimenten op lever (proteïnen) gevoede vliegen wijzen op de noodzaak aan een peptidenfactor die uit de *corpora cardiaca* (CC) wordt vrijgesteld binnen de 4 uur na proteïnenvoeding om zo de volledige proteïnenvertering te garanderen. Manipulaties, uitgevoerd na deze kritisch periode van 4 uur na ingestie van de proteïnerijke maaltijd hebben geen invloed meer op het correct verloop van de volledige maaltijdvertering.

Kwantitatieve differentiële peptidoomanalyse van *corpora cardiaca,* geïsoleerd uit respectievelijk met suiker en lever (5 uur na proteïne voeding) gevoede vliegen toonde een verlaging in de opgeslagen hoeveelheid van het adipokinetisch hormoon. Een injectie van dit neuropeptide in lever gevoede onthoofde vliegen evenals in suiker gevoede intacte vliegen resulteerde dan ook in een dosisafhankelijke verhoging van de middendarm proteolytische activiteit tot het niveau van intacte proteïnen gevoede vliegen. Geen enkel ander peptide, aanwezig in het onderzochte *corpora cardiaca* weefsel, bleek in staat om hetzelfde langetermijn effect te genereren op suikergevoede intacte vliegen en al evenmin

in lever gevoede onthoofde vliegen. Deze resultaten suggereerden een rol van het adipokinethish hormoon bij de verteringsregulatie en dus de voedsel-afhankelijke voortplanting. Bovendien bond dit neurohormoon aan de nieuw geïdentificeerde adipokinetisch hormoonreceptor die tot de familie van G proteïnen-gekoppelde receptoren behoort. De transcripten van de receptor waren het meest abundant in het vetlichaam. Dit was niet verassend aangezien de hoofdfunctie van het adipokinethisch hormoon gelinkt is aan energiemobilisatie en het vetlichaam gekend is als energie-stockerend orgaan. Daarnaast kwam het receptorgen ook sterk tot expressie in de hersenen, de voordarm en de einddarm. Interessant was de vaststelling dat na proteïnenvoeding de hoeveelheid receptortranscript verminderde in bijna alle weefsels. Deze aanpassingen bevorderen wellicht het preferentieel verbruik van via vertering verkregen energiesubstraten om zo de in de weefsels opgeslagen energie te vrijwaren voor later gebruik. Farmacologische karakterisatie gaf aan dat de receptor kon geactiveerd worden door twee vergelijkbare diptere adipokinetisch hormoon liganden met een EC_{50} waarde in het nanomolaire spectrum.

De spijsverteringsactiviteit alsook de ovariële ontwikkeling konden farmacologisch gemanipuleerd worden. Abdominale injectie van 6-hydroxydopamine verhinderde dosisafhankelijk de verhoging van de proteolytische activiteit in de middendarm op het post-transcriptionele niveau en veroorzaakte een vergelijkbaar effect als onthoofding bij levergevoede vliegen. In de vleesvliegen kon die onomkeerbare chemische inhibitie - die wellicht gebeurde door het onderbreken van de hersenen-darm dopaminerge signalering - gebruikt worden als een hulpmiddel voor de gecontroleerde remming van de proteolytische activiteit in de middendarm en de daaraan gekoppelde ovariële ontwikkeling. De remming van de ovariële ontwikkeling was waarschijnlijk een indirect gevolg van een tekort aan circulerende aminozuren die nodig zijn voor de dooiereiwitsynthese.

List of abbreviations

20E	20-hydroxyecdysone
6-OHDA	6-hydroxydopamine
4EPB	4E Binding Protein
AKH	Adipokinetic hormone
AKHR	Adipokinetic hormone receptor
BSA	Bovine serum albumine
CA	Corpora allata
cAMP	cyclic adenosine monophosphate
CC	Corpora cardiaca
CHO cell lines	Chinese hamster ovaries cell lines
DAG	Diacylglycerol
eIF-4E	Eukaryotic translation initiation factor 4E
ER	Endoplasmic reticulum
FG	Frontal ganglion
GPCR	G protein-coupled receptor
HCG	Hypocerebral ganglion
HrTH	Hypertrehalosaemic hormone
IP_3	Inositol triphosphate
JH	Juvenile hormone
LB medium	Luria-Bertani medium
OEH	Ovary ecdysteroidogenic hormone
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PI3 kinase	Phosphatidyloinositol kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
ppf	post protein feeding
PTM	post-translational modification
SNS	Stomatogastric nervous system
TMOF	Trypsin modulating oostatic factor
TOR pathway	Target of rapamycin pathway
TSC1/TSC2	Tuberous sclerosis protein 1 and 2
YPs	Yolk proteins

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CHAPTER 1.

General introduction and aims of the study

1.1 Insects' class

Insects belong to the phylum Arthropoda and constitute the largest and most divergent group of animals living on Earth (Fig. 1.1). Not all species have yet been identified but so far approximately 1.5 million organisms have been examined, classified and named (Chapman, 2013c).

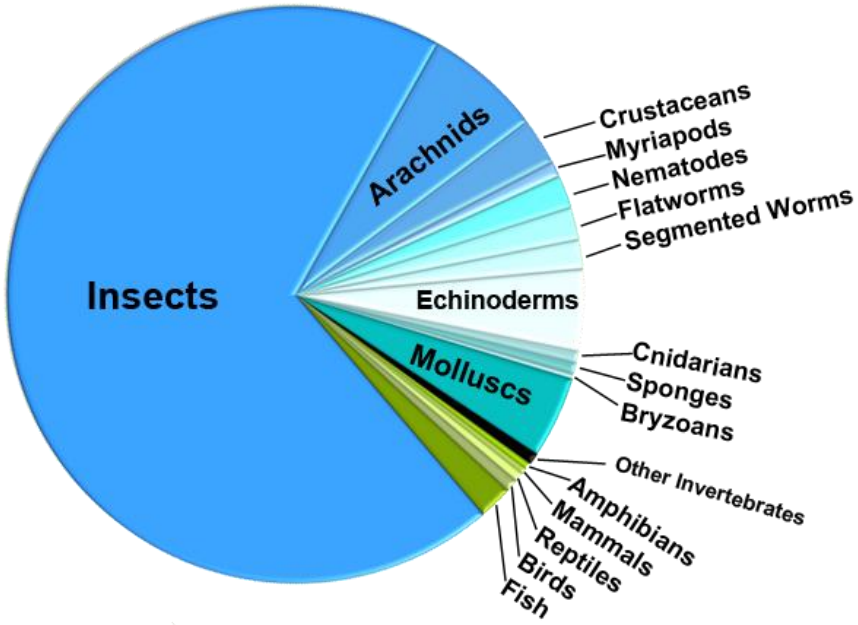

Fig. 1.1 Biodiversity among the animal kingdom. The majority of all living organisms constitutes insects. Image credits: lifegags.com

Insects have well-segmented body parts that consist of a head, thorax and abdomen, and are covered with a relatively hard coating of cuticle. The head contains mouthparts, a single pair of antennae and compound eyes; its main function is perception of external stimuli and feeding. The thorax consists of three segments and carries three pairs of joint legs and usually two pairs of wings; and is the main locomotion center of these organisms.

The abdomen contains multiple segments and holds the gonads, digestion and excretion organs that participate in metabolism, water balance and reproduction.

Insects show huge adaptive potential. Most of them demonstrate flying capacity which gives them some advantage over the rest of the animal kingdom. It influences their dispersal, mating behavior, food seeking and also helps them escape from enemies. Insects are also perfectly able to adjust and live within a huge temperature range and some species can survive at -50^{o}C, while others live in an environment having a temperature over 40^{o}C. Their small size is also beneficial – small organisms require little food, small body size also allows them to colonize even in a small habitat. Another profitable characteristic is their exoskeleton, which is composed of hard regions, separated by soft membranes. This combination assures strength, rigidity but also flexibility that is much more resistant to bending than a vertebrate's endoskeleton. The exoskeleton, the cuticle, is mainly composed of chitin, a polymer of *N*-acetyl-β-d-glucosamine. It works as light but mechanically strong scaffold material and is always associated with cuticle proteins that make up the mechanical properties of the cuticle. Additionally, the outer surface of cuticle is coated with a waxy, water resistant layer that reduces transpiration from the body surface. The respiratory system of insects, characterized by air-transporting tubes, is well suited to these small creatures and as such they are adapted to living on land. Insects that either live their entire life, or only specific developmental stages, in water, are scarce and need special adaptations (Farb, 1964;Imms, 1973b).

Based on their development, insects form three groups: Ametabola, Hemimetabola and Holometabola. Ametabolous insects do not precede any metamorphosis; the adult form results from a progressive increase in size of the larval form. There is no significant difference (except for size and undeveloped genitalia) between larvae and adults. This kind of development takes place in Apterygota (small wingless insects). Hemimetabolous insects go through so-called 'incomplete' metamorphosis which includes three distinct stages: embryo within the egg, subsequent nymph stages and the adult. Nymphs usually resemble adults in their immature stages but their reproductive organs and wings are not fully developed. Complete maturation occurs at the time of adult molt. This kind of development is present in Exopterygota (webspinners, angel insects, earwigs, grasshoppers, crickets, stick insects, cockroaches, termites, mantids, lice and true bugs). Holometabolous insects develop

in four well-defined stages, from embryo through subsequent larval stages into pupa and finally into adult. They undergo 'complete' metamorphosis which may induce the loss of the purely juvenile structures, as well as differentiation of the distinctive adult organs. The larval instars differ significantly from adults and the whole metamorphosis phenomena takes place in the pupal stage. This is observed in all Endopterygota (ants, bees, beetles, twisted-wings parasites, net-veined insects, fleas, flies, mosquitoes, butterflies, moths) (Imms, 1973a;Chapman, 2013b).

1.2 The order of Diptera

Diptera, the true flies order, is the fourth biggest order of the Insecta class, counting at least 122 000 species of worldwide distributed two-winged insects. This order is divided into two suborders: Nematocera (26 families) and Brachycera (104 families). The Nematocera are represented by slightly more primitive 'thread-horned' dipterans with long multi-segmented antennae such as: crane flies, mosquitoes, black flies, midges and fungus gnats. The Brachycera sub-order are the true flies with short (less than six segments) antennae, such as: horse flies, robber flies, fruit flies, houseflies, flesh flies and blow flies. Most dipteran species provide a benefit to the ecosystem through pollination, decomposition or nutrient recycling. Nevertheless, some species have a negative impact on humans and other animals by transmitting diseases such as the malaria (caused by Plasmodium), the sleeping disease (caused by Trypanosomes), the leishmaniosis (caused by Leishmania) as well as the yellow fever, dengue and Zika viruses.

Dipterans have a mobile head, holding a pair of large compound eyes, three ocelli and antennae. The mouthparts, dependent on diet, are adapted for lapping and sponging liquids or for piercing and sucking. In all cases they are built with a labrum, labium and maxillae (Fig. 1.2).

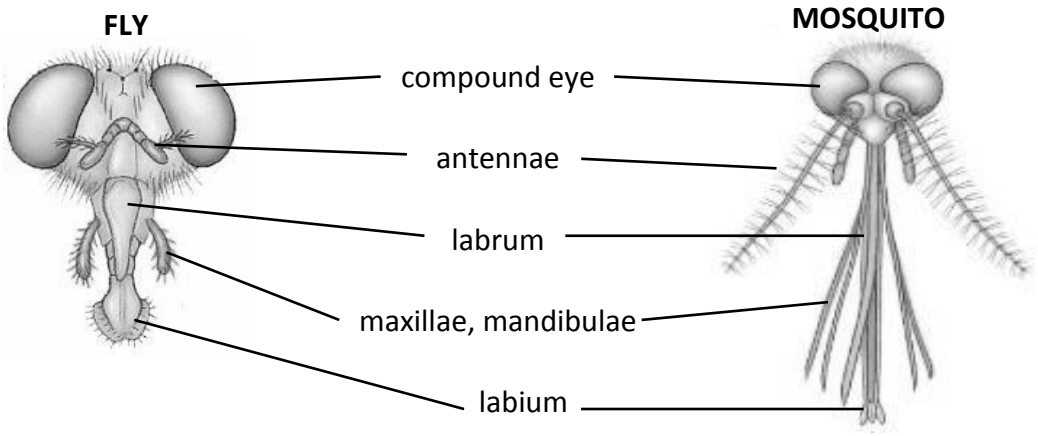

Fig. 1.2 The comparison of the fly lapping and sponging, and the mosquito piercing and sucking mouthparts. The head structures refer to the compound eyes, antennae, labrum, labium, mandibulae and maxillae. Image credits: siera104.com

The specific characteristic of this order is a single pair of big membranous front wings. The hind wings are very much reduced to organs, called halters, and are club-like structured which play a role in flight balancing. The front and the hind segments of the thorax are very small in size, while the middle one, called mesothorax, is enlarged and packed with enormous flight muscles. Wing movement can reach the waving frequency up to 1000 beats per second. Nevertheless there are some exceptional flightless species in this order.

Most dipteran insects are bisexual and parthenogenesis is uncommon. Their mature lifespan is not very long and their main life task is to find a mate and reproduce. The courtship varies between families and species and can involve sound production, dance, nuptial gifts and pheromones. The copulation takes place in the air and the eggs or first larval stages are laid in the environment rich with larval food. Eggs hatch quite fast after being laid, even inside the mother in ovoviviparous species. To assure rapid development and growth, larvae consume as much food as possible in a very short time. Some species are herbivores but most of them feed on dead, organic matter or parasitize other organisms. In general, larval forms such as maggots have no real legs, however some species have developed prolegs to be able to better hold on the difficult habitat surface. They live in aquatic (e.g. mosquito larvae), semi-aquatic or moist environments, as well as in soil, on plants, cadavers and corpses or even in tissue of life animals (see myasis). All dipteran larvae undergo complete

metamorphosis which includes an immobile pupal stage. The pupae itself can vary in form depending on different species (McGavin, 2001;Chapman, 2013c).

1.3 Flesh fly *Sarcophaga crassipalpis* as a model organism

Sarcophaga crassipalpis (Diptera: Sarcophagidae) is a worldwide occurring flesh fly. As its name implies, the life of this fly depends on the flesh of live or dead tissue, to complete its life-cycle. This insect is very much attracted to many types of cadavers including human corpses.

The adult body size fluctuates between 9 – 13 mm. It is light grayish in color with three vertical black strips on the thorax and has a gray-black checkered pattern on its abdomen (Fig. 1.3). The light-gray head contains two large compound eyes with a bright red color, separated by a thick vertical black strip with some golden edges. Both genders can be differentiated by their abdominal shape. Females have a little bit shorter and much more rounded abdomen in contrast to the male abdomens which are longer and thinner. Like many flesh fly species, the end of their abdomens is red-colored in both genders.

Fig. 1.3 Dorsal (left) and lateral (right) view of an adult female flesh fly *Sarcophaga crassipalpis*. Image credits: mattcolephotography.co.uk and rentokil.com.my respectively

Their pupa size oscillates between 5 – 10 mm. Its brown color gets darker, by sclerotization, in accordance with its pupal development.

S. crassipalpis is an ovovivoparous organism, which means that its eggs fully develop and hatch inside the female's uterus and the hatchlings are also stored there till larviposition, which takes place when a proper wounded host or a protein rich substrate is found. Fresh delivered first instar larvae immediately start to eat and develop rapidly. Within an ambient

temperature of 23-24^{o}C, and after approximately five days, larvae achieve the third instar stage and are ready for pupation. Prior to pupation, so-called wandering larvae will leave the humid food supply in search of a suitable dry location. After gut emptying, the now immobile prepupae molt into the pupal stage which still resides within the sclerotized last larval cuticle. The whole metamorphosis of the pupal phase takes about ten days. Flesh flies can show facultative diapause behaviour in the pupal stage which is a kind of hibernation state evoked by any kind of stress condition (temperature, humidity, light, host deficit). During diapause, almost all metabolic processes are greatly reduced to a minimum level, until environmental factors may improve and the animal regain ist normal metabolism (Henrich & Denlinger, 1982;Diaz & Kaufman, 2015).

S. crassipalpis has a natural enemy, a parasitoid wasp called *Nasonia vitripennis*, which specifically target the immobile pupal stage for eggs delivery (Legner, 1995).

This flesh fly is used as a common laboratory model organism in order to study its physiology, biochemistry, endocrinology, reproduction and diapause. The genome is not fully known yet, but there are approximately 9000 published transcripts that are involved in different physiological processes (Hahn *et al.*, 2009;Ragland *et al.*, 2010;Danneels *et al.*, 2013) and nearly complete mitochondrial genome using a 454 sequencing approach (Ramakodi *et al.*, 2015). Additionally, *S. crassipalpis* demonstrates relatively stable anautogenous nature and only serious stress conditions induce the transition into autogeny. Both these facts, the published transcriptome and stable anautogeny, caused that this flesh fly displaced the other flesh fly, *Sarcophaga bullata*, nowadays called *Neobellieria bullata*, which is a more robust specimen but it is very sensitive to any, even small, stress factors and loses its anautogenous characteristic very easily.

1.4 Dipteran reproductive strategy

1.4.1 Anatomy of the female internal reproductive system

The main functions of the female reproductive system are egg production and male spermatozoa storage, until the eggs are ready to be fertilized. The female reproductive

system, similar to vertebrates, is composed of a pair of ovaries. They are connected to a pair of lateral oviducts that are fused together to form the median oviduct, which is connected to the genital chamber of the uterus and terminates with the external opening or vulva. Two types of ectodermal glands open into the uterus: spermathecal and accessory glands (Fig.1.4).

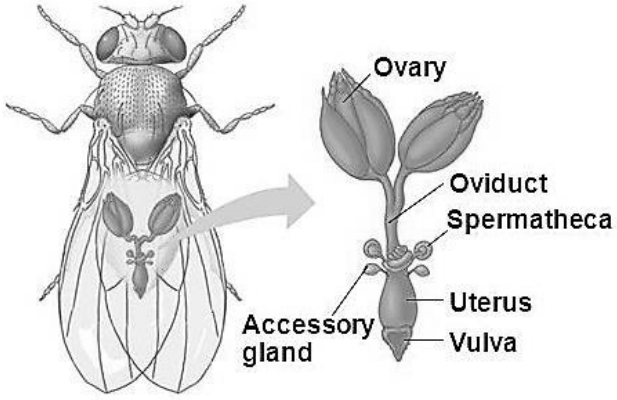

Fig. 1.4 Female fly reproductive system, its organization and location in the abdomen. Image credits: slideplayer.com

The ovaries are situated within the abdomen, above or lateral to the gut. Each of them consists of egg-tubes, termed ovarioles (Fig. 1.5a). An individual ovariole is composed of: a terminal filament (somatic cells); a germarium composed of germ line stem cells; somatic stem cells; cap cells; oogonia and early cysts in which mitosis initiate primary oocyte (Fig.1.4c); a vitellarium in which oocyte grows by yolk deposition and a pedicel. The number of ovarioles varies a lot among different insects, depending on their size, lifestyle or species. Oviducts are tubes with walls, composed of a single layer of cells surrounded by muscles. The median oviduct is usually more muscular than the lateral ducts.

Spermatheca store spermatozoa until the particular moment of egg fertilization. Often, slender ducts of the spermathecal glands are also attached, which provide nourishment to the stored spermatozoa. In case of absence of the spermathecal gland, the necessary nutrients are provided by the glandular cells within the spermatheca. Female accessory glands are also called collateral or cement glands because their secretions enclose and protect eggs or bind them to the substratum during oviposition (Chapman, 2013d).

1.4.2 Oogenesis

The germarium in which oocytes are produced (from oogonia) and the vitellarium in which yolk is accumulated into the oocytes, play very important roles in oocyte growth (Fig 1.5b). The first phase of oocyte growth (in germarium) is regulated by the oocyte's genome itself, while the second phase is mainly controlled by the maternal genes, which encode molecules that are necessary for embryonic development.

Because of the vast diversity among insects, their ovarioles exist in two different forms of either the panoistic form (absence of special nurse cells) or the meroistic form (presence of nurse cells). The meroistic type can be further be divided into telotrophic (nurce cells present only in the germarium part) and polytrophic (nurse cells enclosed within the follicle, present in germarium and vitellarium). The Diptera order is characterized by a meroistic polytrophic type of ovarian development. The germarium consists of prefollicular tissue and stem line oogonia cells that originate directly from germ cells (Fig. 1.5c). After a germ cell divides asymmetrically, one of the daughter cells remains a functional stem cell (attached to the cap cells), whereas the other becomes a precursor cell that divides four times and gives rise to one functional oocyte and 15 accompanying nurse cells.

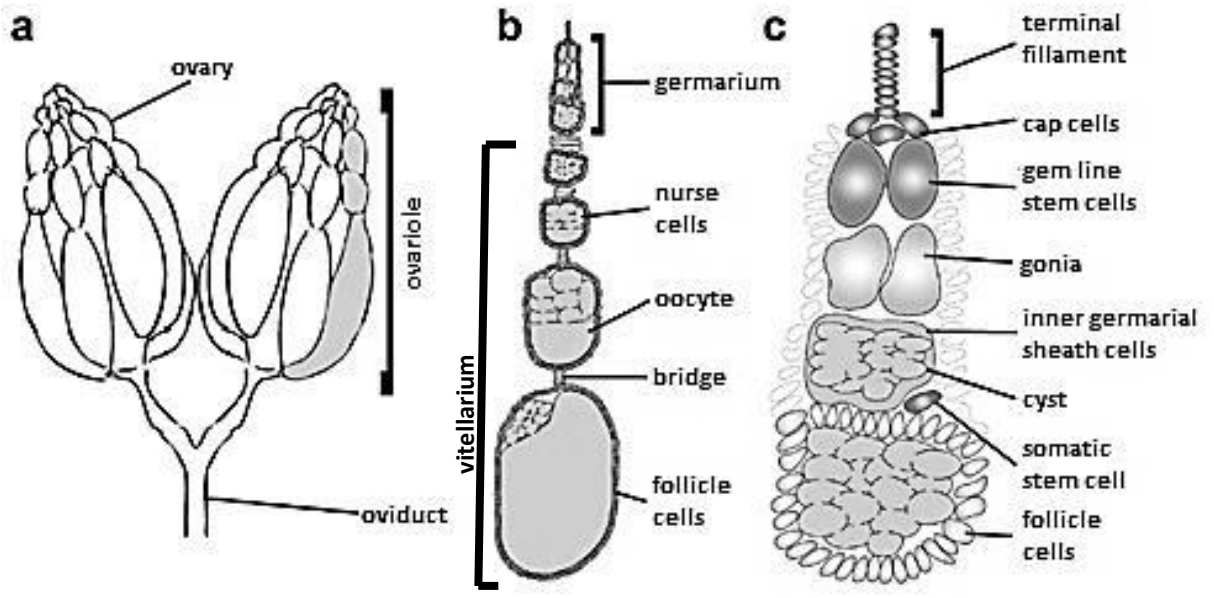

Fig. 1.5 Detailed structure of dipteran ovarian organization. Composition of the ovarioles in the growing ovaries (a). Specific arrangement of the ovariole presenting different egg developmental stages (b). Germarium part of the ovariole showing the initial phase of oogenesis (c). Image credits: (Green *et al.*, 2011)

Before the moment of leaving the germarium, this aggregation of 16 cells is coated with a prefollicular tissue that forms the follicular epithelium. This structure is known as a cyst or egg follicle. Epithelial follicle cell function depends on the oocyte developmental stage - they primarily produce a small amount of yolk proteins and probably also some enzymes that are later involved in yolk processing (Huybrechts *et al.*, 1983;Geysen *et al.*, 1987). Additionally, they also synthesize ecdysone or its precursor. Later, these cells are responsible for the organization of the vitelline envelope. At last they form the chorion of egg shell (Green *et al.*, 2011;Chapman, 2013d).

Due to yolk aggregation in the vitellarium part of the ovariole, the growth of the oocyte is very fast.

1.4.3 Vitellogenesis

A crucial event in insect reproduction is vitellogenesis, which is a large scale production and release of vitellogenins/yolk proteins (YPs) that are immediately sequestrated by developing oocytes. This process can be divided into three stages: previtellogenesis, vitellogenesis and postvitellogenesis (Raikhel *et al.*, 2002).

In the previtellogenic period, the fat body cells (equivalent of a vertebrate liver organ) become competent for large scale synthesis of YPs. Cells storing lipid and glycogen, transform into cells ready for a massive generation of protein. Previtellogenic fat body cells are characterized by large-scale rough endoplasmatic reticulum development and a great number of Golgi complexes (Briers & Huybrechts, 1984;Raikhel, 1987). This remodeling induction is a juvenile hormone (JH) dependent process and the presence of JH stimulates the expression of special receptors that make fat body capable to respond to another steroid hormone, 20-hydroxyecdysone (20E) (Borovsky *et al.*, 1985;Li *et al.*, 2000;Raikhel *et al.*, 2002). All these fat body accommodations close the previtellogenic stage and wait for a signal to start vitellogenesis.

A very low level of YPs synthesis can be observed soon after the adult emergence (Brennan *et al.*, 1982); nevertheless, the real vitellogenesis starts as in the anautogenous mosquito *Aedes aegypti*, upon nutritional stimulation (Van Handel, 1984). In both flies and

mosquitoes, proper nutritional conditions induce the ovaries to release the steroid ecdysone hormone (Fig. 1.6) (Hagedorn *et al.*, 1979). Some studies on *Ae. aegypti* show that a blood meal activated insulin-like peptide is a key player in the stimulation of the ovarian ecdysteroid production (Riehle & Brown, 1999). Upon release, ecdysone is hydroxylated to obtain its active form of 20-hydroxyecdysone (20E) which directly stimulates the fat body to start the transcription and translation of YPs precursors. These precursors are subsequently processed and secreted into the hemolymph from where they are targeted and taken up by the developing oocytes. Most insects produce only one or two vitellogenins, of which the molecular weight varies a lot between different species (210-652 kDa). The exception is the Diptera order in which there are species that produce three to five much smaller yolk polypeptide (YPs) molecules (44-51 kDa). It is interesting to note that abdominal injection of 20E can induce the vitellogenins production, even in the male flesh flies, *Sarcophaga bullata* (Huybrechts & De Loof, 1977;Huybrechts & De Loof, 1982;Briers & Huybrechts, 1984;Raikhel *et al.*, 2002).

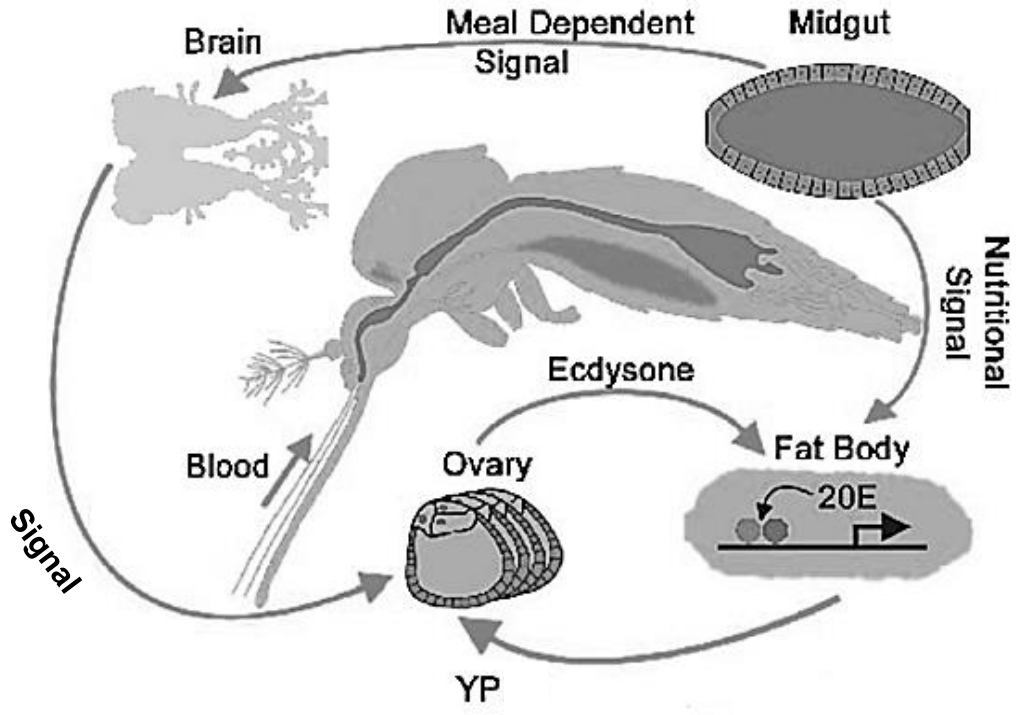

Fig. 1.6 General regulation of vitellogenesis in anautogenous insects. A protein meal-dependent signal inform the brain about changes in the nutritional state of the organism. Subsequent another signal is sent to the ovaries to induce ecdysone production, which following hydroxylation into active 20-hydroxyecdysone stimulates the fat body for YP production that target into the developing ovaries. Abbreviations: 20E, 20-hydroxyecdysone; YP, yolk proteins. Image credits: (Attardo *et al.*, 2005)

The YPs endocytosis into the growing oocytes is a selective process and is mediated by specific receptors, present on the oolemma, the membrane of the oocytes. The receptor-vitellogenin complex is taken up into the oocyte via endocytosis, where the proteins are aggregated and stored in the yolk bodies and the 'empty' receptor is recycled back to the surface membrane. The YPs uptake from the hemolymph is also regulated by JH; it induces particular changes on the surface of the oocytes, called patency. Nevertheless, it does not determine the specificity of the uptake. In many insects, as is the case in *S.crassipalpis*, only the terminal oocyte of each ovariole accumulates vitellogenins or YP's. Hereto , a special factor that blocks YPs uptake by younger follicles is produced (Raikhel & Dhadialla, 1992).

After the termination of the YPs production in the postvitellogenic stage, the fat body regains its nutrient storage and metabolism function until the next vitellogenic cycle (Raikhel *et al.*, 2002)

1.4.4 Anautogenous reproductive strategy and its consequences

Anautogeny is a kind of reproductive strategy that is regulated by the necessity of a proteinaceous meal for the adult female, to be able to fully develop a batch of eggs. This reproductive strategy is typical for many mosquito species and evidently is best studied in those species.

The whole anautogenous characteristic starts at the larval stage of insect development. Nutritional analysis of larvae and pupae of autogenous and anautogenous species of *Culex pipiens* and *Aedes caspius* showed that anautogenous organisms accumulate much smaller quantities of lipids, proteins and carbohydrates that are later passed into the further developmental stages (Soliman *et al.*, 1995). Nevertheless, the duration of the anautogenous larval period does not differ from the one shown by autogenous insects of the same species (Chambers & Klowden, 1994). Anautogeny has a genetic background of different expression levels of larval storage protein genes, distinguished as hexamerins. Anautogenous species express only two out of three known hexamerin genes and the third, missing one appears to

be the female specific gene that plays a key role in nutrient accumulation (Zakharkin *et al.*, 2001).

As a consequence, newly born females emerge with significantly smaller amounts of metabolizable lipids, proteins, carbohydrates, glycogen which represent the energy reserves and building blocks for subsequent oogenesis. The hemolymph free amino acids analysis also showed notable smaller hemolymph titers, especially for arginine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, threonine and valine. Furthermore, not all amino acids present in autogenous insects are detectable in anautogenous mosquitoes. Because of no food-dependent reproduction control in autogenous organisms, these differences disappear during the lifetime of the insect. Anautogenous females also need longer time for their egg development (Soliman *et al.*, 1995;Su & Mulla, 1997a;Su & Mulla, 1997b).

Not only the quantity but also the quality of circulating free amino acids seems to be crucial for YPs production and egg development. Digestion of the protein meal dramatically changes the amino acids concentration in the hemolymph. This high amino acid titer acts as a kind of signal which starts vitellogenesis (Su & Mulla, 1997b). Nevertheless, some circulating molecules appear to be more important than others, as egg development will fail in the absence of one or more crucial amino acids such as leucine, isoleucine, lysine, phenylalanine, threonine, tryptophan, valine, cysteine, arginine and asparagine (Uchida, 1998).

Forced vitellogenesis induction by JH and 20E treatment can stimulate YPs production in anautogenous mosquitoes; nonetheless, almost none of the hormonally-treated females are able to produce fully mature eggs (Khater *et al.*, 1994).

The majority of anautogenous insects is represented by mosquitoes that have a significant social and economic impact in tropical countries. Mosquitoes are very effective disease-spreading vectors. Adult females receive their vitellogenesis-needed nutrients from vertebrates' blood. The repeated blood feeding makes them an efficient instrument for spreading pathogens from host to host. Also, their huge reproductive capacity generates a large number of 'new' vectors in a very short time (Attardo *et al.*, 2005). For instance, in one reproductive cycle, one female of *Aedes aegypti* produces up to 150 eggs that are ready

to be laid three days after blood feeding. One mosquito female can produce up to 500 eggs before she finally dies (Clements, 1992).

The most deadly vector disease is malaria, transmitted by *Anopheles* species. It is estimated that about 3.3 billion people are at risk of the infection. In 2015, the World Health Organization (WHO) estimated about 250 million cases of the malaria disease; including more than 500 000 fatal cases (mostly African children) (WHO, 2014a;Mosquito World, 2015). Very deadly is also yellow fever disease spread by the *Aedes aegypti* mosquito - every year there are approximately 170 000 cases of infection, causing about 60 000 deaths (mainly in Africa). The number of yellow fever transmissions increases due to declining immunity, deforestation, urbanization, population movements and climate changes. Additionally, there is no specific treatment and the medication is only symptomatic, aimed to reduce the symptoms for the comfort of the patient. Nevertheless, the most important preventive measure against yellow fever disease is vaccination, which provides effective immunity within 30 days for 99% of vaccinated people (WHO, 2014b;WHO, 2016a). Another *Ae. aegypti* transmitted disease is Zika virus. It is especially dangerous for pregnant women because of the suspected link between this virus and microcephaly (a neurological disorder that results in babies being born with abnormally small heads and developmental issues). Presently, there is no specific treatment or vaccination available. The best form of the prevention is protection against mosquito bites. Nowadays, the Zika virus is being locally transmitted in the South America and the southern part of the North America. The WHO estimates that three to four million people across both Americas will be infected with the virus in the coming years (WHO, 2016b;Mosquito World, 2016).

1.4.4.1 Factors determining anautogeny

Anautogeny can be an obligate or facultative characteristic determined by either environmental or genetic factors. Some mosquitoes like *Georgecraigius atropalpus* are able to deliver their first batch of eggs without the necessity of a blood meal. Of course there may be some consequences, such as a smaller number of eggs being delivered (Bowen *et al.*, 1994;Gulia-Nuss *et al.*, 2012).

As mentioned before, the determination of the kind of reproductive strategy takes place at the larval stage and is conditioned by multiple factors. Some studies on *Culex tarsalis* identify

temperature as a control agent: higher temperature conditions are less favorable to anautogenous insects (Brust, 1991). Also, duration of photoperiod is significant - long light exposure results in an increased number of autogenous insects in this species (Harwood, 1966). Changes that are caused by these two factors represent a kind of adaptation intended to assure the survival of these mosquitoes during winter time. The normally anautogenous *Aedes aegypti*, which exclusively feeds on human blood, has developed some autogenous strains that live in the jungle, far from their host. In this case, the transition of the reproductive strategy was a kind of survival mechanism due to host deficiency. It was also suggested that host/food availability is one of the strongest determining factors regarding the choice of reproductive strategy (O'Meara & Evans, 1973;Trpis, 1977). On the other hand, poor larval diet and crowded conditions are profitable to maintain anautogenicity in *Culex tarsalis* (Reisen *et al.*, 1986).

From a genetic perspective, the results of crossbreeding experiments between autogenous and anautogenous strains of *Culex pipiens* suggest that there are multiple genes which regulate this type of egg development. The majority of the genes (resulted from insects crossing) associated with the particular egg development strategy determines the type of reproduction (Spielman, 1957).

Considering the fact that a lot of mosquitoes species represent both autogenous and anautogenous specimens and considering their ability to transform from facultative anautogenous insects into autogenous organisms, imply that both anautogenous and autogenous individuals share the same mechanisms of egg development that is regulated by the nutritional state of the organism, as well as by already mentioned environmental and physiological conditions (Attardo *et al.*, 2005).

1.4.5 Vitellogenesis in the anautogenous flesh fly Neobellieria bullata *(*Sarcophaga bullata*)*

Sarcophaga crassipalpis is not an as popular laboratory organism as *Drosophila melanogaster* or *Aedes aegypti*. Consequently the number of scientific publications describing different physiological aspects is also not as high. Nevertheless, the other flesh fly

Neobellieria bullata, formerly called *Sarcophaga bullata*, was a common model organism to study vilellogenesis in the 80's. Both flies, *S. crassipalpis* and *N. bullata*, belong to the Sarcophagidae family and share very high genome similarities. For this reason, all information relating to *N. bullata* reproduction can be useful in this research.

Newborn anautogenous females of *N. bullata,* reveal a very low protein concentration in the hemolymph. They need to consume a minimum of one protein meal to start vitellogenesis and eggs maturation (Wilkens, 1969). Nonetheless, there are always a small amount of circulating YPs present in the hemolymph, even in sugar-fed flies (Schlein, 1977). These YPs can be taken up by oocytes (Huybrechts & De Loof, 1981) but their amount is insufficient for proper egg maturation. Food ingestion and simultaneously increased protein content will stimulate the ovaries to produce and release ecdysone, which subsequently induces the fat body for rapid synthesis and secretion of YPs. A significant increase of vitellogenins concentration was already observed after 4 hours post liver feeding; nevertheless, its apogee was noticed at 24 hours post protein meal and was equal to five multiplicities of the YPs found in sugar-fed insects. There is some balanced constant ratio between newly synthesized and deposited YPs until the end of vitellogenesis, when the entire vitellogenin concentration drops down (Briers & Huybrechts, 1984). Egg maturation takes about 4-5 days; subsequently, ovulated eggs move into the uterus to start embryogenesis, which takes place without any further maternal nutritional support. During pregnancy, the next follicles leave the germarium and become ready for the next vitellogenic cycle (Wilkens, 1968;Engelmann, 1970; Huybrechts & De Loof, 1981).

Results of polyacrylamide gel electrophoresis identified three forms of YPs: 50.0, 53.7 and 56.1 kDa, present in *N. bullata*. None of them are found in male flies, however it is possible to force these males to start YPs synthesis by a simple injection of a physiological dose 20E, ecdysone, makisterone A (potent ecdysone receptor agonist) or muristerone A (phytoecdysteroid analog of ecdysone) (Huybrechts & De Loof, 1977;De Loof *et al.*, 1980;Huybrechts & De Loof, 1982). The same compulsory effect can be obtained in sugar fed female flies. 20E treatment can induce vitellogenesis but due to the insufficient energy reserves, it will not be completed by the production of viable eggs (Huybrechts, 1982). The 20E mode of action also depends on developmental stage, sex and diet of the insect. Liver feeding causes a significant increase of 20E which is observed only in female flies, never

in males, which is probably due to a lack in the need for vitellogenesis in male flies (Briers & De Loof, 1980).

1.4.6 Target of rapamycin (TOR) pathway

Due to the specificity of the anautogenous reproductive strategy, it was highly suggested that there exist some nutritional signals that inform the reproductive tissues/cells of what and how much the organism has eaten. These signals need to be part of the pathways involved in sensing or communicating the nutritional status of the organism and in the initiation of some physiological processes such as growth, cell proliferation or reproduction. One of the possible pathways involved in the transduction of these food dependent signals into the endocrine and reproductive anautogeny systems is the Target of Rapamycin (TOR) pathway (Maestro *et al.*, 2009).

TOR is a serine/threonine kinase present in many eukaryotic organisms. TOR protein is very well conserved in its sequence, especially at its C-terminal end. This high conservation is observed even between evolutionary diverged species (Hansen *et al.*, 2004;Baker & Thummel, 2007). The TOR pathway is stimulated by the presence of amino acids and plays a role in the regulation of transcription and translation of vitellogenins (Hansen *et al.*, 2004).

The TOR pathway combines nutritional and insulin receptor signals (Fig. 1.8). On one hand it is activated by the increasing concentration of free circulating amino acids, especially leucine, detected by amino acid transporters (Attardo *et al.*, 2006). On the other hand, TOR is stimulated by insulin/insulin-like peptides that, by binding to the transmembrane insulin receptors (containing tyrosine kinase domains), cause auto-phosphorylation (Riehle & Brown, 1999). The phosphorylated tyrosine residues stimulate phosphatidylinositol 3 (PI3) kinase which on its turn stimulates the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2) (Alessi & Cohen, 1998). In response to the high level of PIP3, Akt kinase phosphorylates different downstream molecules, inter alia the tuberious sclerosis proteins 1 and 2 complex (TSC1/TSC2) (Avruch *et al.*, 2005).

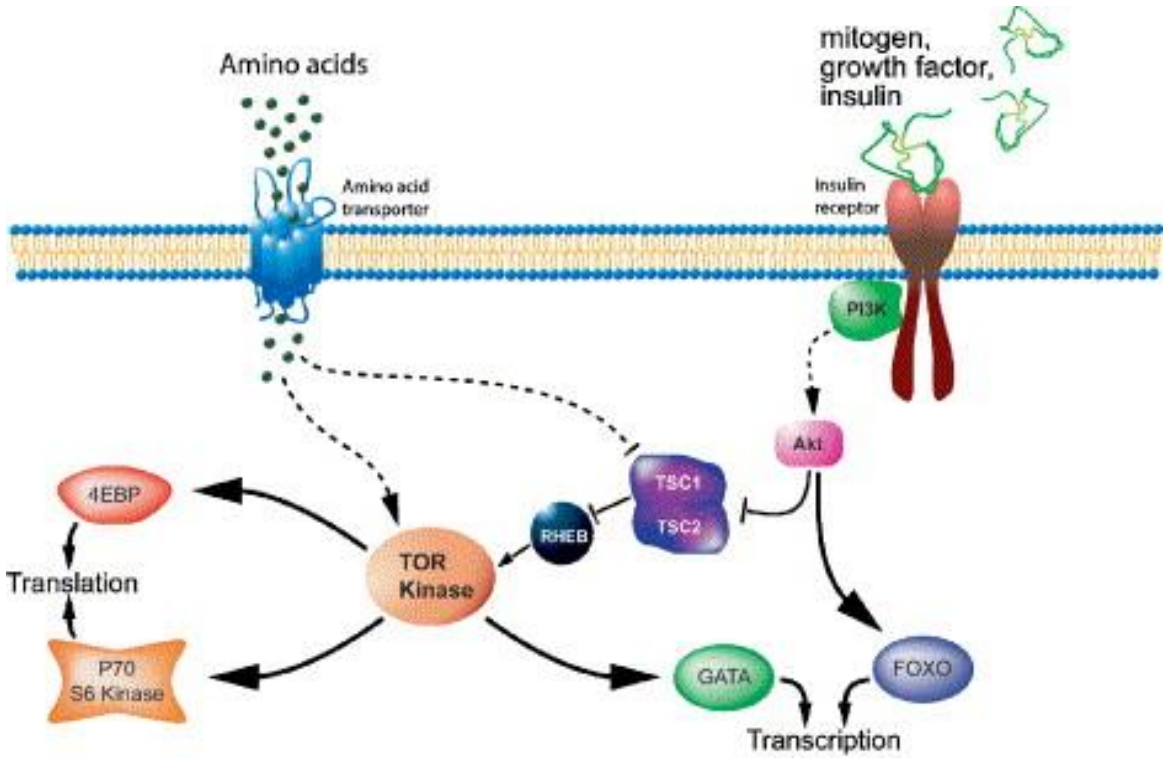

Fig. 1.8 Schematic organization and functioning of the TOR signaling pathway. Pathway activation is stimulated by free amino acids, detected by amino acids transporters and small GTPase Rheb deriving from the insulin receptor signaling pathway. Activated TOR kinase phosphorylates S6 kinase and E4 binding protein allowing for translation of vitellogenins. It also phosphorylates GATA allowing for vitellogenins transcription. Abbreviations: 4EBP, 4E binding protein; Akt, AKT kinase; PI3K, phosphatidylinositol 3 kinase; TOR kinase, target of rapamycin kinase; TSC1/TSC2, tuberous sclerosis protein 1 and 2 complex. Image credits: (Attardo *et al.*, 2005)

Crosstalk between these two pathways occurs via a small GTPase Rheb (Ras homologue enriched in brain), that following its inhibition by TSC1/TSC2 stimulates TOR kinase phosphorylating capability (Stocker *et al.*, 2003). In response to this triggering, TOR kinase phosphorylates and thereby activates two proteins that are involved in the regulation of gene expression. One of them is S6 kinase, which subsequently phosphorylates ribosomal protein S6 that contributes in translation of mRNA containing a 5' polypyrimidine tract. This mRNA structure is characteristic for genes encoding ribosomal proteins, translational elongation factors and growth control proteins (Brown *et al.*, 1995;Jefferies *et al.*, 1997). The second protein that is upregulated by TOR kinase, is the 4E binding protein (4EBP). This factor, in its non-phosphorylated form, binds and inhibits eukaryotic translation initiation

factor E4 (eIF-4E) that participates in cap recognition and recruitment of ribosomes to mRNA. Phosphorylation of 4EPB inactivates it and allows translation (Brunn *et al.*, 1997).

Additionally, TOR kinase also controls the phosphorylation and the nuclear localization of GATA transcriptional factor, called Gln3 (Bertram *et al.*, 2000). The presence of a GATA binding sites is established in the regulatory regions of vitellogenins of *Ae. aegypti* and *D. melanogaster* (Kokoza *et al.*, 2001). Furthermore, one of the Akt kinase targets are FOXO proteins, which are recognized as transcriptional factors in *D. melanogaster* (Kramer *et al.*, 2002).

The TOR pathway can be efficiently down-regulated by the immunosuppressor drug, rapamycin. Experimental incubation of the fat body tissue of the mosquito, *Ae. aegypti*, in a medium containing free amino acids results in YPs expression. The addition of rapamycin into the culture's medium, strongly inhibits this vitellogenin synthesis. Similar inhibiting results were also observed in *in vivo* experiments using RNAi for knocking down the TOR kinase (Hansen et al., 2004).

1.5 Digestion in insects

1.5.1 Structure and functioning of the digestive tract

Insects are a very diverse group in terms of their feeding habits and diets; however all of them share some common characteristics concerning digestion. The digestive tract is responsible for digestion of food and absorption of nutrients that are important for all physiological processes and even essential for the reproduction in anautogenous organisms. The gut is one of the largest organs in an insect's body cavity. Besides its metabolic function, the inner coating also constitutes the first line of defense against various pathogens. It also plays a role in neuronal and endocrine signaling, which regulates nutrient storage or food intake (Lemaitre & Miguel-Aliaga, 2013).

The gut is a compartmentalized organ with very high flexibility and plasticity. It consists of an epithelium layer surrounded by muscles, nerves and trachea. The characteristics and the arrangement of these cells differ a lot, depending on their localization along the gut. In

order to assure its specific function on each level of digestion, this organ is subdivided into a foregut, midgut and hindgut (Fig.1.8). The foregut consists of a pharynx, oesophagus, crop and proventriculus. The proventriculus works as a kind of control valve of food passage into the midgut and it also separates solids and liquids. The midgut is the biggest compartment of this system and is the only digestive organ. Furthermore, the absorption of digested food also takes place in this midgut compartment. In *Drosophila melanogaster*, the midgut is subdivided into six regions with specific metabolic and digestive functions. Some areas are surrounded by muscles which regulate food movement, while others are responsible for the correct folding inside the abdomen. The hindgut is the last compartment of the digestive tract and together with associated Malpighian tubules, functions as an excretory system, which also helps in osmoregulation and water balance. It uses selective secretion and reabsorption of fluids, ions and metabolites, to influence the volume and the composition of the hemolymph.

The epithelium of foregut and hindgut is of ectodermal origin and is coated by an impermeable cuticle; whereas the midgut epithelium is of endodermal origin and in most insects, is lined inside by a peritrophic membrane that divides the midgut into the endoperitrophic and ectoperitrophic space. This provides the recurrent flow of fluid from the posterior back to the anterior part of the midgut, allowing for the reuse of digestive enzymes. The latter has a significant influence on overall digestion efficiency (Gillott, 1980;Bolognesi *et al.*, 2008;Buchon *et al.*,2013).

Gut compartmentalization is attained and maintained by region-specific transcriptional factors (Gillot, 1980;Buchon *et al.*, 2013).

The anatomical organization of the digestive tract of adult flies and larvae differs a lot. This is the consequence of their dietary habits; larvae eat constantly to receive all the nutrients they need for rapid development. Fly maggots are also able to take in solid food, thanks to their chitinaceous mouth hooks. Adult flies eat less frequently and are only able to ingest liquids, via a proboscis. This specific behavior of sporadic feeding by adults can be guaranteed by presence of a crop (a storage organ), which is found only in adult flies (Stoffolano & Haselton, 2013). Because of these differences between insect' developmental stages, the gut is created *de novo* during metamorphosis (Takashima & Hartenstein, 2012).

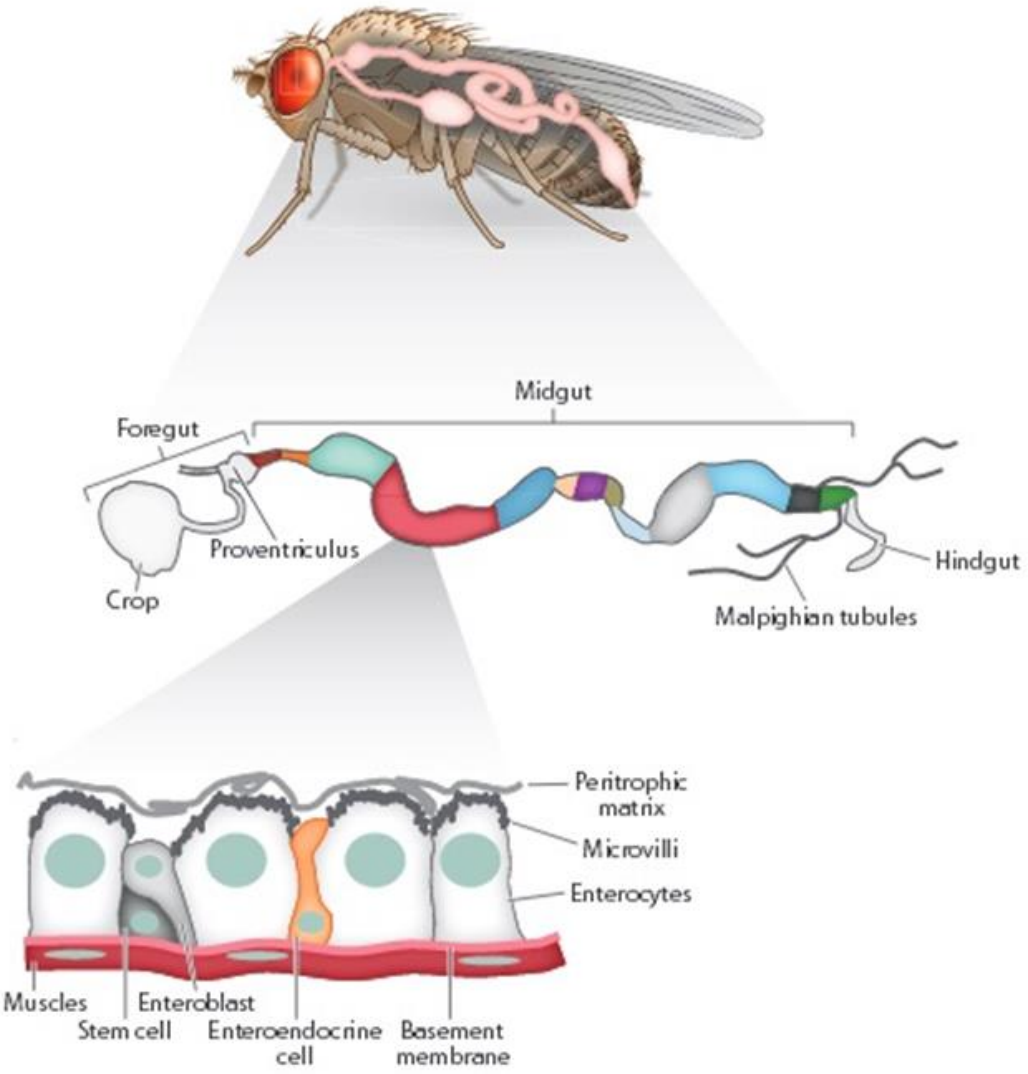

Fig. 1.8 Organization of the digestive tract within the body cavity in *Drosophila melanogaster* (top), its division into three main gut compartments of foregut, midgut with specialized regions and hindgut (middle) and gut cellular composition (bottom). Image credits: (Lemaitre & Miguel-Aliaga, 2013)

The adult gut cells of the foregut, midgut and hindgut are continuously replaced by new cells, which are derived from region-specific regeneration centers of intestinal stem cells, located across the basal surface of the gut epithelium (Takashima *et al.*, 2008;Singh *et al.*, 2011). Stem cells activity is regulated by the overall metabolic state of the insect and by environmental factors. The renewing of these cells is also required in case of intestinal damage, caused by corrosive agents or by pathogenic bacteria (Amcheslavsky *et al.*, 2009;Takashima & Hartenstein, 2012).

1.5.2 Digestive proteolytic enzymes

Digestive enzymes are hydrolases. In insects, they are synthesized and secreted by two types of midgut cells. The first type of constitutively secreting cells constantly produce and immediately release fresh enzymes into the midgut lumen; while the regulated secretory cells synthetize and accumulate digestive proteins, which are released in response to the particular signal (Lehane *et al.*, 1995). Different enzymes act in different ways and on different substrates. Consequently, their classification is established based on the nature of their substrates, their active sites and their cleavage sites.

The digestion of protein food is a complex process, mediated by proteases that hydrolyze peptide bonds. Thanks to the compartmentalized structure of the gut, different steps occur separated from each other. Proteolysis mainly takes place in the endoperitrophic space of midgut and is assured by endoproteases. They digest protein to form oligopeptides that are able to pass the semipermeable peritrophic membrane to the ectoperitrophic space, where the exoproteases further cleave the amino acids residues from both sites. Free amino acids are absorbed by the gut epithelial cells (Terra *et al.*, 1994).

The majority of proteolytic enzymes in the order of Diptera are endopeptidases represented by serine proteases (trypsin and chymotrypsin), which contain a catalytic triad in their active site of histidine, aspartate and serine. Trypsin shows preferential cleavage at the carboxyl side of basic L-amino acids such as arginine or lysine. Its optimum working pH is always alkaline (usually 8-9). Its specificity is similar, but not identical to the trypsin known in vertebrates. Insect trypsin does not require calcium ions for activation or stabilization; it is usually unstable in acidic pH and demonstrates a different sensitivity to natural trypsin inhibitors (Lemos & Terra, 1992;Purcell *et al.*, 1992). Chymotrypsin also acts on the carboxyl side of the targeted amino acid residues, however, it cleaves typically after an aromatic amino acid residue. It requires the same optimal working conditions as insect trypsin. Nevertheless it also differs from the vertebrate's chymotrypsin (Applebaum, 2015).

Trypsin is the main and also the most well-known digestive enzyme in flies. It can be expressed from one or multiple genes. All amino acid sequences show some characteristic motifs of TAAHC, DIA and DSGGP. In carnivorous insects, trypsin occurs in two food-dependent forms of early and late trypsin. Different evidence shows that the synthesis of

early trypsin is constant; in contrast to late trypsin that is under transcriptional regulation (Muller *et al.*, 1993;Borovsky *et al.*, 1996).

The other two groups of insect endopeptidases are cysteine proteases and aspartic proteases with cysteine and respectively, aspartate residues in the active site. There is also a group of exopeptidases which contain metallopeptidases that require a metal ion, such as zinc, for the catalytic process. They cleave starting from the N-terminus (aminopeptidases) or C-terminus (carboxypeptidases) of the substrate (Chapman, 2013a).

1.5.3 Regulatory mechanisms of enzymatic activity

Digestion, as all other physiological processes, needs some steering mechanisms to control the entire action. Because it is a quite complex process, regulation needs to take place at different levels of food intake and is needed for gut motility, enzyme release and nutrient absorption. There are four suggested mechanisms of digestion control: nervous, hormonal, paracrine and prandial (Lehane *et al.*, 1995).

Nervous digestion control is operated by the enteric nervous system that also controls gut movements. Nevertheless the direct nervous control of the enzymatic activity seems to be disputable, due to innervation only being involved in gut motility and its musculature (Dockray, 1988;Lehane *et al.*, 1995).

In hormonal regulation, (neuro-) hormones bind at the outer/hemolymph side of the midgut and influence enzyme release into the gut lumen. They can stimulate the midgut in direct or indirect way. This control mechanism has been very well studied in the mosquito *Aedes aegypti*. Mosquito females that have mature or nearly mature eggs are no longer able to digest blood meal (Detinova, 1962). This down-streaming effect is caused by a decapeptide called trypsin modulating oostatic factor (AeTMOF) (Borovsky *et al.*, 1990). A very strong inhibiting function of NebTMOF (of up to 90% reduction) was also observed in the flesh fly *Neobelleria bullata* (Borovsky *et al.*, 1996).

The insect's midgut epithelium contains a large amount of endocrine cells involved in paracrine control. Based on their morphology, these cells form two groups of open and close

cells. Open cells are in direct contact with gut lumen, via microvilli, and respond directly to food substances that are present in the midgut lumen (Leite & Evangelista, 2001). Woodring et al. (2009) suggest that in crickets, *Gryllus bimaculatus*, glucose and maltose bind to the receptors of gut endocrine cells and induce the release of paracrine (allatostatin 5) into the hemolymph, that bind to receptors of nearby enzyme-producing cells and induce amylase release.

In the prandial control mechanism (also called secretogogue control), the ingestion of specific food components can stimulate cells to synthesize and secrete digestive enzymes. It was observed that female stable flies*, Stomoxys calcitrans*, that were fed with whole blood, blood serum or plasma, demonstrated a great increase in midgut trypsin activity. This is in contrast to the other group of flies fed with red blood cells and that increased their trypsin activity level only by a little (Houseman *et al.*, 1988).

However, it is very difficult to design experiments that separate the paracrine from the prandial responses, which means that not a lot is known about either of these mechanisms (Lehane *et al.*, 1995).

Most of these regulatory mechanisms involve peptide hormones which makes the process of digestion the easiest and most potent for pharmacological manipulation. This is also the most promising direction for the creation of new insecticides and therefore a good knowledge of the exact neuropeptidergic regulation of digestion is required.

1.5.4 Neuropeptidergic regulation of feeding and digestion

Food uptake and transport along the digestive system is regulated by the central nervous and stomatogastric nervous systems (SNS), which consist of a number of ganglia that are connected to each other and to the central nervous system. SNS can vary in different insects but in general it follows some particular structural patterns.

Starting at the beginning of the digestive tract, the mouthparts are controlled by the sub oesophageal ganglion that is composed of fused ganglia (derived of the mandibular, maxillary and labial segments) and which is situated below the brain. It is connected to

the brain via a pair of circum oesophageal connectives and it innervates the mandibles, maxillae, labium, hypopharynx, salivary glands and neck muscles (Gillott, 1980).

The frontal ganglion (FG) is localized in the center of the digestive tract. It lies on the dorsal site of the oesophagus and is linked to the brain via a pair of nerves called frontal connectives. The FG is also connected, by a large recurrent nerve, to the hypocerebral ganglion (HCG) which itself is linked to the corpora cardiaca. The HCG system is connected with the proventricular ganglion by the oesophageal nerve (Fig.1.8) (Hartenstein, 1997).

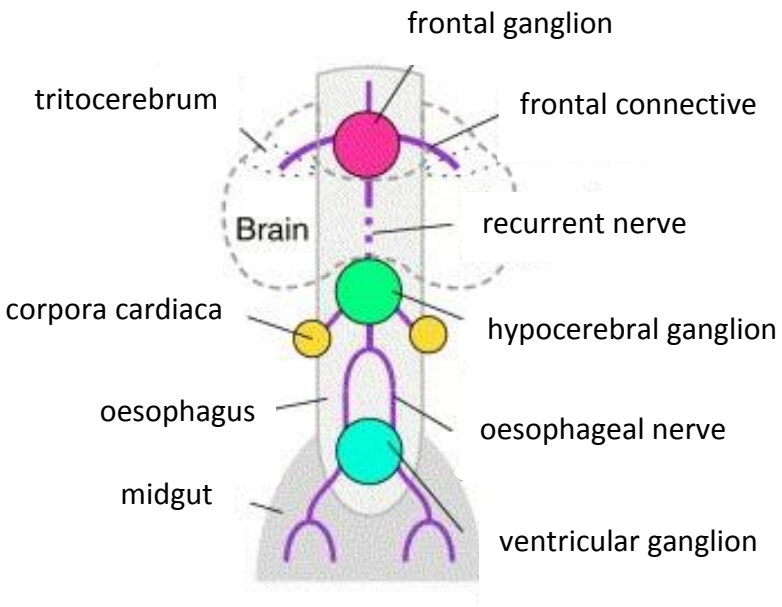

Fig. 1.8 General organization of the stomatogastric nervous system in insects. The frontal ganglion is connected to the hypercerebral ganglion, by the recurrent nerve. Furthermore, this hypercerebral ganglion is connected to the (pro)ventricular ganglion, by the oesophageal nerve. Image credits: cell.com

Nowadays, it is known that SNS is involved in the foregut motor activity which is associated with food intake, via the FG and stimulates the peristaltic contractions of the foregut. The removal of this ganglion results in food accumulation in the crop (Schoofs & Spiess, 2007). In addition, the FG generated contraction frequencies, depend on the amount of food that is actually present in the crop (Ayali & Zilberstein, 2004).

The hindgut innervation is derived from proctodeal and rectal nerves controlled by the terminal abdominal ganglion, which is under control of the central nervous system (Copenhaver, 2007).

Digestion as a process is controlled at multiple levels, starting from food intake through gut motility, enzyme synthesis/release and nutrient absorption. Many neuropeptides show myoactivity regulation of the visceral muscles by contracting and relaxing the gut muscles that are essential for food motility and enzyme flow. Nevertheless, some of them show inhibiting effects while others work as stimulators. These peptides seem to be widely distributed in the central and peripheral nervous systems. The specific localization of neurons and nerve axons in different parts of the gut strongly indicate their role in the regulation of feeding and digestion. A lot of these peptides were found in the FG, other ganglia or nerves of SNS when using immunocytochemical techniques (reviewed by Audsley & Weaver, 2009).

1.5.4.1 Trypsin modulating oostatic factor (TMOF)

TMOF is the best studied peptide which regulates digestion in anautogenous insects. A decapeptide form was initially isolated from the ovaries of the mosquito, *Aedes aegypti*, (Borovsky et al., 1990) whereas a hexapeptide was found in the flesh fly, *Neobelliera bullata* (Bylemans *et al.*, 1994). TMOF is synthesized and secreted from the ovaries (follicular epithelium) into the hemolymph after the protein meal digestion. It binds the specific midgut epithelial cell receptor and terminates trypsin synthesis by translational control. As mentioned before, in anautogenous insects, trypsin occurs in two food dependent forms of early and late trypsin. TMOF injection into flesh flies or mosquitoes does not affect early trypsin gene expression but inhibits the translation of the late trypsin mRNA (Borovsky *et al.*, 1996).

This is species specific - the flesh fly TMOF shares no structural similarities with the mosquito TMOF and does not inhibit the mosquito trypsin synthesis (reviewed by Borovsky, 2003). In mosquitoes, the TMOF action is noticed at 18 hours post blood feeding.

TMOF is also a potential insecticide. Blocking the trypsin biosynthesis results is a shortage of free amino acids, downregulates larval growth and development, as well as blocks

vitellogenin production which consequently terminates the oocytes growth in the adult insects (Borovsky *et al.*,1996;Borovsky & Meola, 2004).

1.5.4.2 FMRFamides and FMRFamides-like peptides

The true FMRFamides, being tetrapeptides, were first identified in the sunray venus clam *Macrocallista nimbosain* (Price & Greenburg, 1977). Insect FMRFamides-like peptides are longer, N-terminally extended peptides, containing the conserved C-terminal RFamide end. There are at least three subfamilies of these peptides present in insects: the FMRFamides, the HMRFamides and FLRFamides (Orchard *et al.*, 2001).

In the blood feeding bug, *Rhodnius prolixus*, FMRFamides-like peptides are observed in different parts of SNS such as FG, HCG, recurrent and oesophageal nerves, and in the corpora cardiaca-corpora allata (CC-CA) complex. These peptides increase the frequency of the foregut contractions and are secreted into the hemolymph after blood feeding, which strongly suggests their function in feeding/digestion (Tsang & Orchard, 1991;Elia *et al.*, 1993). In the flesh fly, *Neobelleria bullata*, FMRFamides-like peptides are detected in HCG, proventriculus neurons and neurons along the surface of midgut epithelium, but also in the endocrine cells of the midgut which again indicates their feeding and gut peristalsis regulatory role (Sivasubramanian, 1992). They are also observed in neurons innervating the anterior and posterior parts of the digestive tract in the silkworm, *Bombyx mori* (Na *et al.*, 2004).

The first insect peptide containing a FLRFamide C-terminus was discovered in the Madeira cockroach, *Leucophaea maderae,* and was called (leuco)myosuppressin because of its ability to inhibit hindgut contractions (Holman *et al.*, 1986). In the locust, *Schistocerca gregaria*, these peptides are present in some part of the SNS (frontal, suboesophageal and abdominal ganglions), which suggests their role in feeding/digestion regulation (Clynen & Schoofs, 2009). In another locust, *Locusta migratoria*, myosuppressins not only inhibit midgut contractions but also increase enzymatic activity (Hill & Orchard, 2005).

The extended HMRFamide peptide was first isolated from the cockroach, *Leucophaea maderae*, as it demonstrated the ability to stimulate hindgut muscle contractions (Nachman *et al.*, 1986). These peptides are designated as sulfakinins because of their sulphated

tyrosine residue that is essential for their biological activity. In the locust, *S. gregaria*, sulfakinins perform a strong anti-feeding action resulting in reduced food uptake. In another locust, *L. migratoria*, sulfakinins not only reduce food uptake but also reduce digestive enzyme secretion. Additionally, these experiments also proved the significance of the sulphate group, since the non-sulphated peptide displayed no effect (Wei *et al.*, 2000;Zels *et al.*, 2015). A similar anti-feedant effect is observed in the blow fly, *Phormia regina* (Downer *et al.*, 2007) and in the cockroach, *Blatella germanica* (Maestro *et al.*, 2001), where sulfokinins also stimulate foregut and hindgut contractions.

1.5.4.3 FXPLRamides related peptides

FXPLRamide related peptides, also called pyrokinins, were originally isolated from cockroach, *Leucophaea maderae* (Holman *et al.*, 1986). In another cockroach, *Periplaneta americana*, pyrokinins are synthesized in the suboesophageal ganglion and tritocerebrum and stimulate the foregut, hindgut and oviduct contractions (Predel *et al.*, 1999;Predel & Nachman, 2001).

1.5.4.4 Kinins

First insect kinins, also called myokinins or leucokinins, were discovered in the cockroach *L. maderae* (Holman *et al.*, 1986) as myotropic peptides acting on hindgut. They are characterized by their specific C-terminus of FXXWGamide. They are well known in different insects for their myotropic stimulation, however in the house cricket, *Acheta domesticus*, they also show some potential diuretic activity, that works differently from diuretic hormone (Coast *et al.*, 1990). In the caterpillar, *Oposina arenosella*, leucokinins inhibit the release of enzymes into the gut lumen (Harshini *et al.*, 2002). In the blood-sucking bug, *Rhodnius prolixus*, kinins are very widely distributed in the central nervous and digestive systems as hindgut, posterior midgut and midgut endocrine cells, which indicates their role in digestion/feeding behaviour (Te Brugge *et al.*, 2001).

1.5.4.5 Allatoregulatory peptides

Allatoregulatory peptides were identified by their inhibitory and stimulatory effects on JH synthesis, dividing them respectively into allatostatins and allatotropins. There are three types of allatostatins (A, B and C) and two types of structurally unrelated allatotropins. They

are characterized by their myoactive effect on the insect gut (reviewed by Audsley & Weaver, 2009).

The A-type allatostatins were originally identified in the cockroach, *Diploptera punctata*, as peptides inhibiting JH synthesis (Pratt *et al.*, 1989). They are characterized by the very well conserved C-terminal end and its last five amino acids that are crucial for the biological activity of the peptide (F/YXFGLamide), while their N-terminus can vary a lot among different insects (Weaver *et al.*, 1998). In the codling moth, *Cydia pomolnella*, these peptides are innervating the foregut via two large neurons, which originate from the FG (Duve *et al.*, 1995). In the noctuid moth, *Helicoverpa armigera*, they are also detected within the neurons that have their axons within the recurrent nerve. These axons pass along the crop prior to further branching to control the crop muscles and stomadeal valve. These axons also project to the anterior part of the midgut and terminate in the longitudinal muscles; nevertheless, two other axons continue along the whole length of the midgut up to the proctodeal valve (Duve *et al.*, 1999). The A-type allatostatins are detected in the midgut endocrine cells in the locust, *Locusta migratoria* (Robertson & Lange, 2010). In the cricket *Gryllus bimaculatus*, these peptides are released from the gut endocrine cells (in the response to food intake) and bind to caecal cells, stimulating trypsin and amylase activity (Woodring *et al.*, 2009). A similar enzyme secretion stimulating effect is observed in the cockroach *Diploptera punctate*, where these peptides influence the carbohydrase synthesis and release (Fúse *et al.*, 1999).

The B-type allatostatins were first discovered in the cricket, *G. bimaculatus*, and are characterized by their specific sequence motif of $W(X)_6$Wamide (Blackburn *et al.*, 1995;Lorenz *et al.*, 1995). However, in fact, the first peptide from this family was originally isolated from the head of the locust, *Locusta migratoria*, (Schoofs *et al.*, 1991) in which it was named Locusta myoinhibiting peptide seen its suppressing effect on the hindgut and oviduct contractions. In cockroach, *P. americana*, the B-type allatostatins are localized in the FG, HCG, the oesophageal nerve and the neurons innervating the crop muscles and they inhibit foregut contractions (Predel *et al.*, 2001). In the blood feeding bug, *Rhodnius prolixus*, these peptides are found within neurons in the central nervous system and within the innervation network of the salivary glands, hindgut and reproductive system of both male and female. Bugs treated with B-type allatostatins display a decreased hindgut

contraction frequency (Lange *et al.*, 2012). These peptides also strongly inhibit foregut and hindgut contractions and are able to inhibit the food intake in the German cockroach, *Blatella germanica* (Aguilar *et al.*, 2006).

The last group of the C-type allatostatins were found and characterized from the tobacco hornworn, *Manduca sexta*. They are characterized by non-amidated, N-terminally blocked peptides of 15 amino acids, which contain a disulphide bridge between residues 7 and 14 and have a well conserved PISCF motif (Kramer *et al.*, 1991). Similar to the previous B-type allatostatins, they also show inhibitory regulation of the gut contractions and feeding behavior in two lepidopteran species, *Lacanobia oleracea* and *Spodoptera littoralis* (Matthews *et al.*, 2008). Nevertheless, in the red flour beetle, *Tribolium castaneum*, this peptide stimulates the secretion of proteases into the midgut lumen (Audsley *et al.*, 2013).

The first type of allatotropins was originally found in the tobacco hornworn, *M. sexta* (Kataoka *et al.*, 1989), whereas the second type was identified in the fall armyworm, *Spodoptera frugiperda* (Abdel-Latief *et al.*, 2004). In the locusts, *L. migratoria*, they stimulate midgut and oviduct contractions (Paemen *et al.*, 1991). In the kissing bug, *Triatoma infestans*, these peptides are present in brain, CC-CA complex, Malpghian tubules, midgut, midgut endocrine cells and aorta (Riccillo & Ronderos, 2010), which implies their role in the control of feeding/digestion. Increased gut contractions are also observed in the red flour beetle, *T. castaneum*, and in the desert locust, *S. gregaria,* treated with allatotropin-like peptide (Vuerinckx *et al.*, 2011;Lismont *et al.*, 2015).

In general, all allatoregulatory peptides are found in the FG and within the gut (foregut or the whole gut) muscles innervation, which strongly suggests their role in peristalsis by the regulation (inhibition or stimulation respectively) of the frequency and the amplitude of the peristalsis contractions (reviewed by Audsley & Weaver, 2009). It is suggested that in response to the information derived from the food intake and from the gut, the allatoregulatory peptides regulate gut passage (by inhibitory and excitatory mechanisms) according to the specific phases of digestion and absorption (Duve *et al.*, 2000). Interestingly, the allatoregulatory peptides interactions as studied in the moth, *Lacanobia oleracea*, demonstrate that the allatostatin effects are dominant over the allatotropin stimulation. The inhibition of the gut motility caused by allatostatins cannot be reversed by

allatotropins. However the allatotropin action can be stopped by allatostatins. The reversable inhibition is only observed with A-type allatostatins (Matthews *et al.*, 2007).

1.5.4.6 Neuropeptide F and short neuropeptide F

The name neuropeptide F (amidated phenylalanine end) comes from the vertebrate neuropeptide Y (amidated tyrosine end), which plays an important role in the regulation of homeostasis by the control of the food intake (reviewed by Chee & Colmers, 2008). In the fruit fly, *D. melanogaster*, this peptide consists of 36 amino acids and due to its length is often called long neuropeptide F, to distinguish it from the so-called short neuropeptides F. This long peptide is detected in the brain and endocrine cells of the midgut, which indicates its role in feeding and digestion (Brown *et al.*, 1999). In the mosquito, *Ae. aegypti*, long neuropeptide F is present in the brain, suboesophageal ganglion and midgut and its hemolymph titer is highest just before and 24 hours post feeding (Stanek *et al.*, 2002). In the blood sucking bug, *R. prolixus*, this peptide is released into the hemolymph post blood meal (Gonzalez & Orchard, 2008).

Short neuropeptide F-like peptides were first found and studied in the Colorado potato beetle, *Leptinotarsa decemlineata* (Spittaels *et al.*, 1996). In the *D. melanogaster* genome, four predicted forms (ending with RLRFamide or RLRWamide) of short neuropeptides F are found and experiments proved their main role in feeding behavior as an appetite or food searching stimulating peptide (Vanden Broeck, 2001;Lee *et al.*, 2004;Root *et al.*, 2011). Similar, stimulatory effects are also observed in silkworm, *B. mori* (Nagata *et al.*, 2011). Nevertheless, in some other insects, like mosquito, *Ae. aegypti,* or black blow fly, *P. regina,* short neuropeptide F displays a completely opposite, feeding inhibitory behavior (Brown *et al.*, 1994;reviewed by Spit *et al.*, 2012). Antifeedant properties of these peptides are also observed in the desert locust, *S. gregaria*. Short neuropeptide F gene expression depends on availability of nutrients in the hemolymph and is decreased in the starved locusts, forcing them for food searching and feeding (Dillen *et al.*, 2013;Dillen *et al.*, 2014;Dillen *et al.*, 2016). However, some other authors claim the opposite, stimulatory feeding effect of this peptide in desert locust (Van Wielendaele *et al.*, 2013).

1.5.4.7 Proctolin

Proctolin was first found in the cockroach *Periplaneta americana* (Starratt & Brown, 1975) as a pentapeptide which stimulates hindgut contractions. In locust, *L. migratoria*, these peptides are present in the central, peripheral and stomatogastric nervous systems, and CC. They are detected within the innervation of every gut compartment, which suggests its role in the control of feeding/digestion (Clark *et al.*, 2006). Some studies provide evidence about proctolins upregulating the foregut, midgut and hindgut contractions in the cockroach, *D. punctata* and in the locusts, *L. migratoria* and *S. gregaria* (Fúse & Orchard, 1998;Lange & Orchard, 1998;Gray *et al.*, 2000). Noteworthy, in the Lepidoptera order, some authors claim the absence of this proctolin regulatory mechanism (Konopińska & Rosiński, 1999;Nagata *et al.*, 2011).

1.5.4.8 Tachykinin related peptides

Tachykinin related peptides (also designated as insect tachykinins) are small peptides characterized by three C-terminal motifs of FXG/AXRamide or (more vertebrate related) FXGLMamide (reviewed by Spit *et al.*, 2012). First tachykinin related peptides were identified in the locust, *L. migratoria* (Schoofs *et al.*, 1990a;Schoofs *et al.*, 1990b), and showed some myotropic properties on all gut part and oviduct. In the cockroach, *Leucophaea maderae*, tachykinin related peptides are localized in the central nervous system, SNS, gut innervation and midgut endocrine cells. They are released into the hemolymph upon starvation conditions. It is suggested that their stimulating effect upon gut contraction improve food motility, digestive enzymes flow and consecutive nutrient absorption. Additionally, some of these peptides show strong diuretic activity (Johard *et al.*, 2003;Winther & Nässel, 2001). Insect tachykinins influence food intake in the cockroach, *B. germanica*, and in the silkworm, *B. mori* (Pascual *et al.*, 2008;Nagata *et al.*, 2011).

1.5.4.9 Diuretic hormone

Diuretic hormones stimulate fluid secretion by Malpighian tubules. Surprisingly, they also have some influence on feeding behavior. Injection of this peptide into the lepidopteran *Heliothis virescens* results in the reduction of food consumption and water excretion. High doses of diuretic hormones will down regulate the diuretic hormone receptors in

the Malpighian tubules and result in suppression of liquid secretion. This inhibition prevents excessive water redemption from moisture-rich food that, in consequence, down regulates the feeding processes, especially the feeding duration (Keeley *et al.*, 1992). Similar effect on reduced food consumption is observed in the tobacco hornworm, *M. sexta*, and in the desert locust, *S. gregaria* (Ma *et al.*, 2000;Van Wielendaele *et al.*, 2012). Authors explain it by one, or a combination, of the potential mechanisms of the peptide being 1) antifeedant activity, 2) reduction of food assimilation, 3) suppression of the digestion and absorption processes in the midgut or 4) disruption of diuresis.

1.5.4.10 Adipokinetic hormone

The adipokinetic hormone (AKH) was first isolated from the locust, *Locusta migratoria* (Stone *et al.,* 1976). It is a member of a large group of structurally related peptides of the AKH/red pigment-concentrating hormone (AKH/RPCH) family. AKH is one of the most abundant neuropeptides in most insects. It is synthesized and stored in the neuroendocrine gland known as *corpora cardiaca* (CC) and it usually consists of eight to ten amino acids (Gäde, 2009). All of the AKH peptids found to date, share some common structural features of a pyroglutamic acid blocked N-terminal end, an aromatic amino acid at the fourth position, a tryptophan at the eight position and an amided C-terminal end (Weaver *et al.*, 2012;Marco *et al.*, 2011). Many insects among which some Diptera such as *Lucilia cuprina* and *Neobelleria bullata* (Rahman *et al.,* 2013) have only one form of functional AKH peptide. The locust, *Schistocerca gregaria* (Candy, 2002), cockroach *Periplaneta americana* (Hansen *et al.*, 2006) and silkworm *Bombyx mori* (Roller et al., 2008) have two functional AKHs and the locust, *Locusta migratoria,* has three forms of biologically active AKHs (Diederen *et al.*, 2002).

AKH is primarily involved in the mobilization of stored energy-carrying molecules to support all high-energetic processes, for example flying (Van der Horst, 2003), by activating glycogen phosphorylase or triacylglycerol lipase in the fat body. Based on the specificity of the increased energetic molecules in the hemolymph, AKH can be found in the literature under different names. Peptides that increase the concentration of lipids are usually called AKH while similar peptides that stimulates carbohydrates, especially the hemolymph trehalose concentration, are called hypertrehalosemic hormone and the corresponding

peptides that stimulate proline increase, are known as hyperprolinaemic hormone (reviewed by Gäde *et al.*, 1997). Nevertheless, despite of all these nomenclature differences and reference molecules, they all represent similar neuropeptides having the AKH sequence. Importantly, the role of AKH is not only limited to metabolism: it acts on flight muscles which influence the speed of flight (Goldsworthy *et al.*, 1979); it effects walking activity in non-flying insects (Kodrik *et al.*, 2000); it stimulates the frequency of heart beats (Scarborough *et al.*, 1984); it participates in immune responses (Goldsworthy *et al.*, 2005); it moderates stress reactions (Kodrik, 2008;Kodrik *et al.*, 2005); and it is involved in egg development (Abdel-Latief & Hoffmann, 2007;Lorenz, 2003).

Additionally, in the firebug, *Pyrrhocoris apterus*, AKH stimulates gut locomotory activity, as well as enzymatic activity in the midgut and salivary glands (Kodrik *et al.*, 2000;Kodrik *et al.*, 2012;Vinokurov *et al.*, 2014). Also increased amylase activity is observed in AKH treated cockroach, *P. americana* (Bodláková *et al.*, 2016).

1.6 Aims of the study

Sarcophagidae, belonging to the flesh fly family can be harmful for human and life stock. Flies of the *Sarcophaga* genus mostly breed on excrement, carrion and other decaying organic material. Nevertheless, sometimes they can be involved in myiasis which means the infestation of wounds of living vertebrates with dipterous maggots which feed on the host tissue for at least a certain period of their life. The other genus of this family, *Wohlfahrtia* (especially *Wohlfahrtia magnifica),* is an obligate parasite of warm-blooded vertebrates in Europe and Africa. Larviparous females of these insects deposit about 120-170 larvae near wounds or body openings of humans and other animals such as sheep, goats, cattle, horses, donkeys, pigs, dogs, camels and geese. Their larvae feed and mature in a few days and then leave the wound for pupation. Some *Wohlfahrtia* species can even directly penetrate the host's skin and produce furuncles.

Myiasis can be also caused by many other flies of Calliphoridae family (*Calliphora, Chrysomya, Cochliomyia, Lucilia, Phormia* and *Protophormia* species), which also share a protein-dependent reproductive strategy (fao.org, 2015).

Especially, the myiasis caused by *Lucilia sericata* is a serious problem for Belgian sheep farmers. The number of farming units affected by this infection increased from 4% reported in 2001 to 61% established in 2006 (vlaanderen.be, 2008).

As in the case of anautogenous blood meal-dependent mosquitoes, flesh flies and blow flies have been massively exposed to so many different pesticides over the years that many species have developed a high resistance to most of the insecticide classes in use. In many cases, a successful control technology for these dipteran adults is simply not available or is often uneconomical. This forces us to create new, much more specific and low budget solutions. Therefore, a proper understanding of anautogeny regulating mechanisms is very important to limit the transmission of these diseases. One important control strategy might target their protein meal-dependent reproduction. Hitherto, to start, a detailed understanding of the digestion process (and its regulation) is crucial to reduce the population of these blood/meat-dependent insects.

Our study was mainly focused on the fundamental research aspects of anautogeny regulation in flesh flies and tried to add new insights to the present understanding of this reproductive strategy. Based on the current knowledge obtained from mosquito species, we tried to implement our flesh fly model to study this phenomenon. Since anautogenous flesh flies, *Sarcophaga crassipalpis*, reveal some practical advantages over mosquitoes (such as they do not need a blood meal) which made them a much safer and more convenient model organism to work with. Also, their significantly bigger size allowed for more precise manipulations, like dissections of tiny organs such as *corpora cardiaca* or repeated microinjections.

To better understand 'building blocks' and basic principles of the regulation of anautogenicity, we started our work with the identification and characterization of the digestive enzymes present and active within the midgut of the flesh fly. All changes in proteolytic activity, elicited by the protein meal intake by the adult female fly, were examined at both mRNA and protein level and compared to the existing information concerning the blood meal-induced changes in the mosquito digestive activity. Nevertheless, the main part of this research was focused towards the regulatory mechanisms of the so-called gut-brain axis, that control the protein meal digestion and consequently reproduction

in these anautogenous insects. All objectives of this study can be summarized with the following research questions:

- Which enzymes are responsible for protein digestion in flesh flies?
- What kind of control mechanism regulates the changes in digestion after the protein meal? What factors/signals are responsible for starting these changes?
- Which particular factor(s) stimulates proteolytic digestive enzyme activity?
- Do flesh flies synthesize a gonadotrophin related to the ovarian ecdysteroidogenic hormone (OEH) (known to be released in mosquitoes in response to the protein meal)?
- Can we control (inhibit/enhance) midgut digestive activity and consequently control ovarian development in anautogenous flesh flies?

CHAPTER 2.

Characterization of digestive proteolytic activity and its regulation in the anautogenous flesh fly, *Sarcophaga crassipalpis*‡

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Bil,M.; Huybrechts,R. (2016) Pharmacological regulation of digestion in the anautogenous flesh fly, *Sarcophaga crassipalpis*, by simple injection of 6-hydroxydopamine. Archives of Insect Biochemistry and Physiology 91, 137-151.

2.1 Introduction

Feeding and digestion are very complex processes that combine physical, chemical and nutritional signaling. Their regulation is comprised of positive and negative sensory feedbacks, distention of the gut, effects of nutrients and the release of peptides or hormones from/to the gut or brain (Wei *et al.*, 2000). The act of feeding causes the release of different signaling molecules centrally from the brain and CC (neurohormones, neurotransmitters or neuromodulators), as well as locally from the SNS and/or SOG (neuromodulators or neurotransmitters). These signaling peptides support ingestion, digestion, movement and absorption of food through the gut. Furthermore, they also regulate the volume and composition of the insect hemolymph following feeding related processes (reviewed by Audsley & Weaver, 2009).

Based on the organization of the digestive system, insects can be divided into two groups. The first group includes continuous digesters; in these insects, food storage, digestion and nutrient absorption takes place in different parts along the length of the gut. Their gut epithelium cells (responsible for enzyme biosynthesis and endocrine regulation of the process) are usually morphologically and functionally divergent in different compartments. The other group consists of batch digesters, which is strongly represented by

adult mosquitoes, where each individual blood meal is digested over the entire surface of the midgut at the same time. Their midgut epithelium cells do not differentiate much. During the digestion process, there are significant changes in the level of midgut enzymatic activity that are especially noticeable in the batch digesters. This strongly indicates that the synthesis and secretion of digestive enzymes are regulated during the digestive cycle (Blakemore *et al.*, 1995).

In analogy with anautogenous mosquitoes, anautogenous adult female flesh flies, *Sarcophaga crassipalpis*, need to feed on an additional proteinaceous meal that is digested and used as a source of energy and building blocks during vitellogenesis. In this species, not much is known about the midgut digestive activity, which is a crucial process in this food-dependent reproductive strategy. Therefore, in the following chapters, I will try to explain this process, its nature and control mechanisms. This chapter started with the characterization of midgut digestive activity with the main focus on protein digestion in the midgut. Changes in enzymatic activity, induced by the protein meal intake were examined at the total proteolytic activity level, and also at the detailed level of the activity of particular enzymes. We also discussed some of the first steps that were taken to understand what kind of control mechanisms are involved in regulation of digestion in this insect species.

2.2 Material and methods

2.2.1 Insect rearing

The anautogenous flesh fly, *Sarcophaga crassipalpis*, was bred under specific laboratory conditions of 23^{o}C and a light/dark cycle of 14/10 hours, as described by Denlinger (1972). Due to the general scientific purpose of studying the neuropeptidergic regulation of digestion and subsequent ovarian development, only adult female flies of 4 days after emergence were used for the experiments. This age assures that the flies had completed their previtellogenic development and are fully capable to start vitellogenesis and oocytes development. Also to avoid any variation between different populations, each experiment was performed using insects that were housed in the same cage, having the same age. Flies

in cages selected for experiments were kept on a sugar and water diet only to ensure no earlier increase of digestive activity and subsequent development of their ovaries. The other flies, in cages that were not used for any scientific aims, were fed with additional pieces of cow liver to continue normal egg development and larviposition to preserve the culture.

2.2.2 Midgut dissection

Right before dissection, flies were sedated with carbon dioxide and subsequently immobilized using a needle on a Petri dish containing a silicon polymer, mixed with active coal powder. To avoid any degradation or sticking of the tissue to the surface of the polymer, the whole manipulation took place under sterile *Sarcophaga* Ringer's solution containing 121.5 mM NaCl; 10 mM KCl; 1 mM NaH_2PO_4; 10 mM $NaHCO_3$; 0.7 mM $MgCl_2$; 2.2 mM $CaCl_2$; pH 6.8 (Meulemans & De Loof, 1992). The operations were performed using a binocular microscope with two external cold light sources on both sides and using ethanol/acetone disinfected forceps.

2.2.3 Preparation of tissue samples for microscopy

Dissected midguts of both, sugar only, as well as sugar and liver fed flies were treated overnight in a 0.1 M phosphate-buffered saline (PBS) solution, containing 4% paraformaldehyde. Afterwards, they were rinsed with PBS and water to remove all salt. Subsequently, the tissues were colored for one minute in 30 µl Mayers Hematoxylin that colored the cell nucleus and neurons cytoplasm in blue (to facilitate the manipulation of the small-sized tissues). Colored midguts were incubated overnight in 70% ethanol and then during the day in 95% and 100% ethanol and again incubated overnight in 100% ethanol mixed with xylol in 1:1 proportions. Next, the organs were embedded in paraffin and sliced into 6µm sections. Deparaffinated dry preparations were once again fixed in a 0.1 M PBS solution containing 4% paraformaldehyde for 30 minutes, and subsequently rinsed with water and ethanol. Next, they were incubated in chloroform for 10 minutes and again rinsed with ethanol and water. Afterwards, tissues were once again colored with Mayers Hematoxylin for seven minutes and eosin for three minutes (coloring the cell cytoplasm). All excessive coloring compounds were rinsed from the tissue section with water which was then dehydrated with an alcohol series and xylol prior to closing with Depex.

2.2.4 Feeding and decapitation procedures

All female flies selected for experiments, were placed in small transparent cages; the maximum number of insects per cage was 30. Flies that were kept on a sugar-only diet, later called sugar-fed flies, had unlimited access to sugar and water. Other flies, called liver-fed flies, were offered a single protein meal of a small piece of cow liver (served on a plastic plate). The smell of the meat attracted the flies, which immediately started eating. The feeding duration was 45 minutes and recording of this time started when once 50% of the selected flies began their consumption. On all graphical visualizations, the beginning of the feeding referred to 0 hour post protein feeding on the time (x) axis of all experiments concerning
the digestive activity changes under particular conditions. After 45 minutes, the liver was removed and the flies only had access to water and sugar.

Liver consumption was additionally checked during the midgut dissection. Flies that did not take in any protein meal, or consumed only very little volumes, were excluded from the experiments.

In case of decapitation necessity (to eliminate any brain control participation), the fly head was cut off in the 'neck part' between head and thorax, by the use of ethanol/acetone disinfected micro scissors. The post-decapitation wound was sealed with a drop of melted wax to avoid hemolymph leakage and overall dehydration.

Prior to the midgut dissection, decapitated flies were checked for vital signs by touching their legs with a needle. The legs movement in response to touching, indicated vital state of the organism. Flies that did not respond to touching, were considered dead and eliminated from the experiments.

2.2.5 Determination of midgut proteolytic activity

Proteolytic activity was measured by the amount of azocasein digested by the gut homogenate and this in the presence or absence of specific protease inhibitors (Bylemans *et al.*, 1994). Each measured data point represented four independent biological replicas, wherefore each individual replica comprised a pool of three midguts (to avoid large variation between samples of the same condition). All midguts were dissected in Ringer's solution and

immediately transferred into Eppendorf tubes containing 600 μl of the same solution. Afterwards, the samples were homogenized to release enzymes from gut content into the solution. Eighty five μl of the homogenates were transferred to new tubes containing either 15 μl correction buffer (50 mM Tris-HCl pH 7-8; 10 mM $CaCl_2$) or 15 μl specific protease inhibitor. Those mixtures were incubated with 100 μl 1% azocasein for 45 minutes at room temperature. The digestive reaction was stopped by adding 75 μl 10% cold trichloroacetic acid and the amount of the digested substrate was measured by spectrophotometer at a 405 nm wavelength. The resulting absorbance was normalized against the midgut weight of the samples. Because there was no possibility of generating standard curves, the results of the enzymatic activity were relative compared to each other. Negative control flies were decapitated immediately post liver meal priming in order to suppress the release of the digestion stimulating brain factor.

The additional comparison of the results obtained for the gut homogenates and the solutions containing the secreted enzymes (three dissected midguts of liver-fed flies incubated in 600 μl Ringer's solution for 30 minutes) is presented in the supplementary data (Supp. fig. 2.1).

The following protease-specific inhibitors were used: *Schistocerca gregaria* pacifastin inhibitor 1 and 2 (Simonet *et al.*, 2003) for inhibition of trypsin and chymotrypsin respectively, E-64 (Sigma-Aldrich) and cystatin (Sigma-Aldrich) for cysteine protease inhibition and a potato carboxypeptidase inhibitor (Sigma-Aldrich) for Zinc metallo carboxypeptidase inhibition. Each protease-specific inhibitor was used in 15 μl solution with a concentration of 25 μM.

The same procedure was used to determine trypsin and chymotrypsin activity changes post protein feeding, by the use of 100 μl of the enzyme-specific substrates (1 mM) of N-benzoyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide (BPVApNA) (Sigma-Aldrich) and succinyl-L-ala-ala-pro-l-phenylalanine-p-nitroanilide (SAApNA) (Sigma-Aldrich) respectively.

2.2.6 RNA isolation and cDNA synthesis

Dissected midguts were immediately transferred into special tubes containing MagNa Lyser Green Beads (Roche) and frozen in liquid nitrogen. Afterwards all collected samples were

homogenized using the MagNa Lyzer (Roche). Total RNA was isolated from the tissue homogenates using RNeasy Lipid Tissue Mini kit (Qiagen) supplemented with the optional procedure of the DNase treatment (RNase-free DNase set, Qiagen) preventing any genomic DNA contamination.

The quality and quantity of the obtained RNA was verified on the NanoDrop (Thermo Fisher Scientific Inc.). Equal amounts of 1 µg RNA of each sample was reverse transcribed by using SuperScript III Reverse Transcriptase (Invitrogen) and random hexamer primers as described in the providers protocol. The obtained cDNA was diluted tenfold before starting the experiment.

2.2.7 Transcriptome analysis

The genome of *S. crassipalpis* is unfortunately unsequenced. Nevertheless, there is some published genomic information in a form of transcriptome of 207,110 records with an average read length of 241 nucleotides. These reads are assembled into 20,995 contigs and 31,056 singletons (Hahn *et al*., 2009). The screening of the *in silico* translated nucleotide database using a protein query of an early trypsin amino acid sequence of *Aedes aegypti* (GenBank Acc. No. **AAM34268.1**), was used to find all possible trypsin sequences. Those nucleotide sequences were later translated into amino acid sequences using the online Expasy translation tool (http://web.expasy.org/translate/).

In order to determine if the identified cDNA's encode early or late trypsin, several amino acid sequences of other insects recognized as early and late trypsins were aligned, using the online ClustalO tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) and analyzed for specific early and late trypsin motifs (Supp. fig. 2.3). Those motifs were then searched for in the sequences of *S. crassipalpis*.

The amino acid sequences of early trypsin derived from *Aedes aegypti* (GenBank Acc. No. **AAM34268.1**), *Anopheles gambiae* (GenBank Acc. No. **CAA80517.1**) and *Culex quinquefasciatus* (GenBank Acc. No. **AAK50138.1**). The sequences of late trypsin derived from *Aedes aegypti* (GenBank Acc. No. **AAF82286.1**), *Culex quinquefasciatus* (GenBank Acc. No. **AAB37261.1**) and *Ochlerotatus epactius* (GenBank Acc. No **AAN75000.1**) (Supp. fig. 2.4).

2.2.8 Trypsin gene expression

Transcript levels of different trypsin genes were measured using quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR). To obtain trustworthy results of the measurement, eight flesh fly specific housekeeping genes (elongation factor 1α (GenBank Acc. No. **GQ409484.1**), heat shock protein 90 (GenBank Acc. No. **AF261773.1**), heat shock protein 70 (GenBank Acc. No. **AF107338.2**), glyceraldehyde 3-phosphate dehydrogenase (GenBank Acc. No. **EZ598085.1**), ribosomal protein S18 (GenBank Acc. No. **GQ409208.1**), ribosomal protein L32 (Hahn transcriptome: **HAHN.FLY.cl.345**), tubulin (Hahn transcriptome: **EZ599932.1**) and actin (Hanh transcriptome: **EUA37Q301ATGMZ**)) were tested for the most stable endogenous expression in the two experimental conditions of sugar-only and sugar and liver-fed flies. Using qbase+ software v.2.4 based on geNorm algorithm (Vandesompele *et al.*, 2002) three genes were determined to be the most stably expressed and were used as the reference genes (tubulin, ribosomal protein S18 and heat shock protein 90) (Supp. fig. 2.2) All primers were designed by Primer Express software v.2.0 (Applied Biosystems) and ordered from Sigma-Aldrich Company (Table 2.1).

Gene	Forward primer sequence	Reverse primer sequence
EF1α	5'-GTCTTCGCCCCCGCTAAC-3'	5'-AGCTTCGTGGTGCATTTCAAC-3'
HSP90	5'-GATGCCGACAAGAAAGATAAAGA-3'	5'-CGGGTCCAGATGGGCTTAGT-3'
HSP70	5'-CGGGCTAAGCGAACTTTGTCT-3'	5'-TCCCTCGAAAAGAGCATCGA-3'
GAPDH	5'-TCCTACGATGCCATCAAGGC-3'	5'-ACGAAATCGGTGGAGACGAC-3'
RPS18	5'-TATGGCTCTCTCAGTCGCTTCC-3'	5'-TGGTGCCCTTCCGTCAATT-3'
RPL32	5'-AATTGCCCATGGTGTTTCCTC-3'	5'-TGTGAACGAACACGACCATTG-3'
TUB	5'-CGGAAACTAGCTGATCAATGCA-3'	5'-ACCACCAAAGGAATGGAACACT-3'
ACT	5'-AACGCAAATACTCCGTCTGGAT-3'	5'-CGGGACCAGATTCGTCGTA-3'
ETR1	5'-TTCTGGTGGCCCATTGGTA-3'	5'-CATAGCCCCAGGAGACAACAC-3'
ETR2	5'-ACTGCCGCTCATTGCTTACA-3'	5'-GCCGGCACGAACTTTTAAAA-3'
ETR3	5'-CCATTGAAGCTCATCCCTATCAG-3'	5'-GGAACCACCACAGAAATGGAA-3'
LTR	5'-TGCCAAAAATGGATGGTAAATACTC-3'	5'-CCAACCGGAAGCCCAAA-3'

Table 2.1. Primer sequences used for the qRT-PCR experiment. Abbreviations: EF1α, elongation factor 1α; HSP90, heat shock protein 90; HSP70, heat shock protein 70; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPS18, ribosomal protein S18; RPL32, ribosomal protein L32; TUB, tubulin; ACT, actin; ETR, trypsin; LTR, late trypsin.

Each PCR mixture contained 10µl Fast SYBR Green Master Mix (Applied Biosystems), 1 µl forward and 1 µl reverse primers (10 µM), 3 µl MQ and 5 µl DNA. The PCR reactions were performed in duplicate on a StepOne Plus System (Applied Biosystems) using the temperatures program of 95 °C for 10 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The relative expression quantities of the genes were calculated using the $\Delta\Delta$Ct method (Livak & Schmittgen, 2001).

2.2.9 Peptide extraction

*Corpora cardiac*as, a part of the retrocerebral complex, were dissected in Ringer's solution. Organs were collected from either sugar-fed flies or from protein-fed flies at different time points post feeding. The dissected and cleaned tissue was immediately transferred into acidic methanol (methanol/water/trifluoroacetic acid in proportion of 90/9/1). Tissue samples were first centrifuged and then sonicated, filtrated on a spin down 0.22 µm filter and dried using SpeedVac. LoBind Microcentrifuge Tubes (Eppendorf) were used to avoid the peptides sticking to the tube wall. The samples were additionally prepurified on C18 ZipTip pipette tips (Millipore) and dried. The extracts meant for injection were resolubilized in Fly Ringer. Recipient flies were given 2 µl injections. To assure maximum response and correct comparison with the protein fed condition, the recipient flies, unless otherwise stated, were primed by a time-limited protein meal and subsequently decapitated (see paragraph 2.2.4.)

2.2.10 Dose-response experiment

To define the active amounts of CC extract, flies were fed with liver for 30 minutes, decapitated and subsequently injected with different equivalents of CC extract. The evoked increase in digestive activity was measured at 4 hours post protein feeding (the moment of the highest proteolytic activity). Each particular amount of CC extract was tested on ten individual flies.

2.2.11 Statistical analysis

All statistical analyses were performed with the use of Statistica 12 software. The differences between groups were determined by factorial ANOVA to analyze the variance involving two independent variables (time point and treatment) on the dependent variable (proteolytic

activity). After the global analysis, the differences between particular time points of each group pairs were determined by the post-hoc Tukey test.

Dose-response experiments were validated by the student's t-test by comparison of all different conditions to the positive control of non-treated liver-fed flies, in order to find the most accurate treatment for further experiment.

2.3 Results

2.3.1 Midgut morphology

Microscopic preparations of the midguts of sugar (Fig. 2.1A-C) and liver (Fig.2.1D-E) fed flies did not show any significant difference between those two dietary conditions, except for the presence of liver content in the gut lumen. No significant enlargement of the gut diameter was observed, though stretching of the midgut epithelium in the liver-fed condition was obvious, as the epithelium lining was clearly thicker in sugar-fed compared to liver-fed flies.

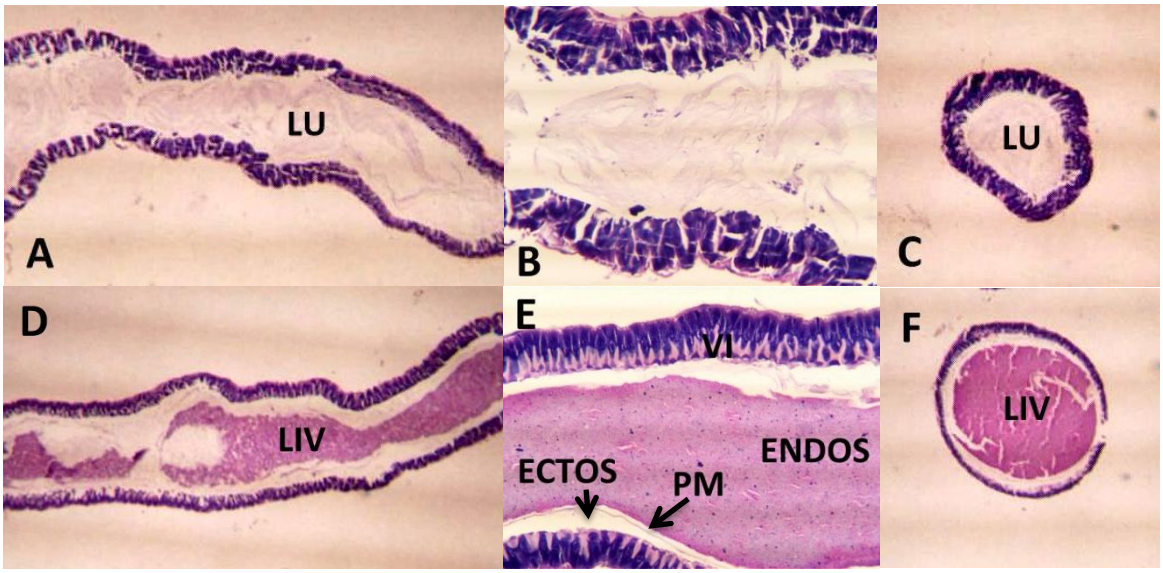


Fig. 2.1 Microscopic preparations of the midguts of sugar (A-C) and liver (D-F) fed flies. Sagittal sections of 4X and 20X magnifications (A, D and respectively B, E); transverse sections of 4X magnifications (C, F). No notable changes except for the presence of the liver content and much more stretched and regular epithelium in liver fed insects. Abbreviations: ECTOS, ectotrophic space; ENDOS, endotrophic space; LIV, liver content; LU, midgut lumen; PM, peritrophic membrane.

Some gut structures like villus or crypt depths could be easily observed. Preparations made on liver-fed insects showed the very well visible peritrophic membrane surrounding the midgut content. This membrane separates the endoperitrophic space containing ingested food from ectoperitrophic space, situated between the dividing membrane and the gut villi. Irregularity of the midgut diameter (wider and narrower diameters) suggested peristaltic movement of the alimentary canal.

2.3.2 Changes in midgut proteolytic activity post protein or amino acids meal

Females of anautogenous flesh flies, *Sarcophaga crassipalpis*, that were bread under strict sugar and water diet showed relatively low but constant secretion of serine proteases in their midguts. No difference between day and night time collected samples was observed. Midgut total proteolytic activity of liver-fed flies increased after the consumption of the protein meal (Fig. 2.2).

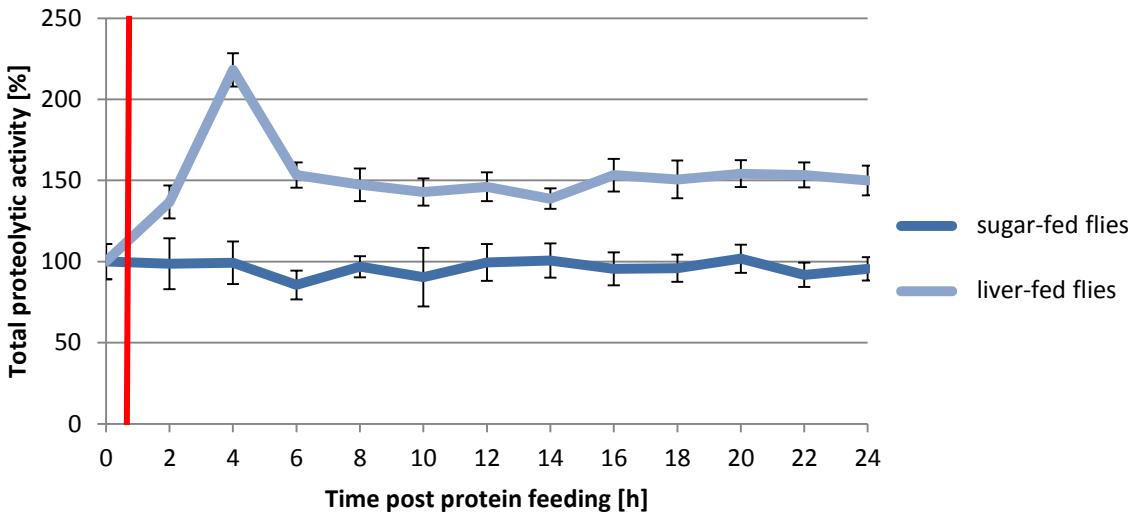


Fig. 2.2 Changes in midgut proteolytic activity of sugar and liver-fed flies measured up to 24 hours ppf. Proteolytic activity of sugar-fed flies presents constant level of the enzymatic secretion, in contrast to liver-fed flies, where the midgut proteolytic digestion increases after liver feeding. Red line indicates the end of feeding moment(four biological replicates of three pooled midguts each); mean *± SD*.

The first significant changes (p_{2h}=0.00399) were observed at 2 hours post protein feeding (ppf) when the enzymatic activity reached the level of about 140% of the basic sugar fed flies' digestion. A maximum peak of the proteolytic activity was observed at 4 hours ppf and

surpasses the basal level by 214% (p_{4h}=0.00019). Thereafter, the overall proteolytic activity decreased slightly but still remained on a level of 140-150% compared to the level in sugar-fed flies ($p_{6\text{-}24h}$=0.00399). That elevated activity persisted up to 24 hours ppf after which no further observations were made.

Nevertheless, feeding flies with a free amino acid solution, devoid of any protein, (3 ml IPL-41 Insect Medium, Sigma-Aldrich for 45 minutes) did not increase the enzymatic activity in their midgut (Fig. 2.3). Statistical analysis confirmed the lack of significant difference between proteolytic digestion of sugar and amino acid-fed flies.

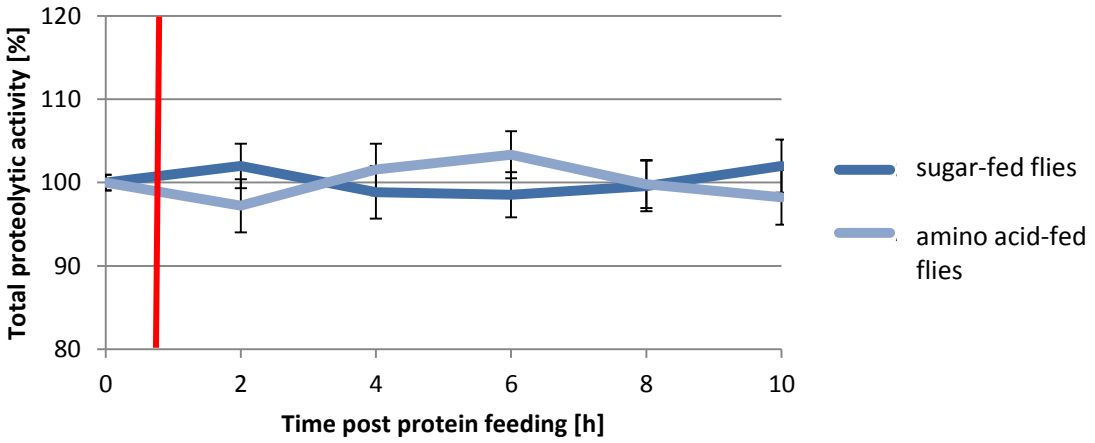


Fig. 2.3 Proteolytic activity profile of sugar and free amino acid-fed flies. No significant difference is observed between both dietary conditions. Red line indicates the end of feeding moment (four biological replicates of three pooled midguts each); mean ± *SD*.

2.3.3 Characterization of the proteolytic enzymes involved in digestion

Analysis of the use of specific proteases inhibitors revealed that half of all proteolytic enzyme activity in sugar-fed flies was represented by trypsin (Fig. 2.4A). The second biggest contributors, responsible for almost one third of the activity, were metallo carboxypeptidases. The others, smaller parts of 11% and 6% were represented by chymotrypsin and cysteine proteases respectively.

Following liver feeding, at the peak level of proteolytic activity, trypsin (in relative terms) still represented the most important protease because it was responsible for half of the measured proteolytic activity (Fig. 2.4B). At that moment, where digestion was the most intensive, chymotrypsin and cysteine protease activity proportionally increased 2.5 folds, up

to respectively 28% and 11% of the global measured proteolytic activity. Metallo carboxypeptidases activity represented only 1% of the azocasein-measured enzymatic activity at that particular moment.

Similar results were also observed in decapitated liver-fed flies (Fig. 2.4C), measured 4 hours ppf. In headless flies, trypsin was still the most abundant proteolytic enzyme, maintaining more than a 50% contribution. Similar to non-decapitated liver-fed flies, roughly two folds increase in chymotrypsin and cysteine protease activity was noted.

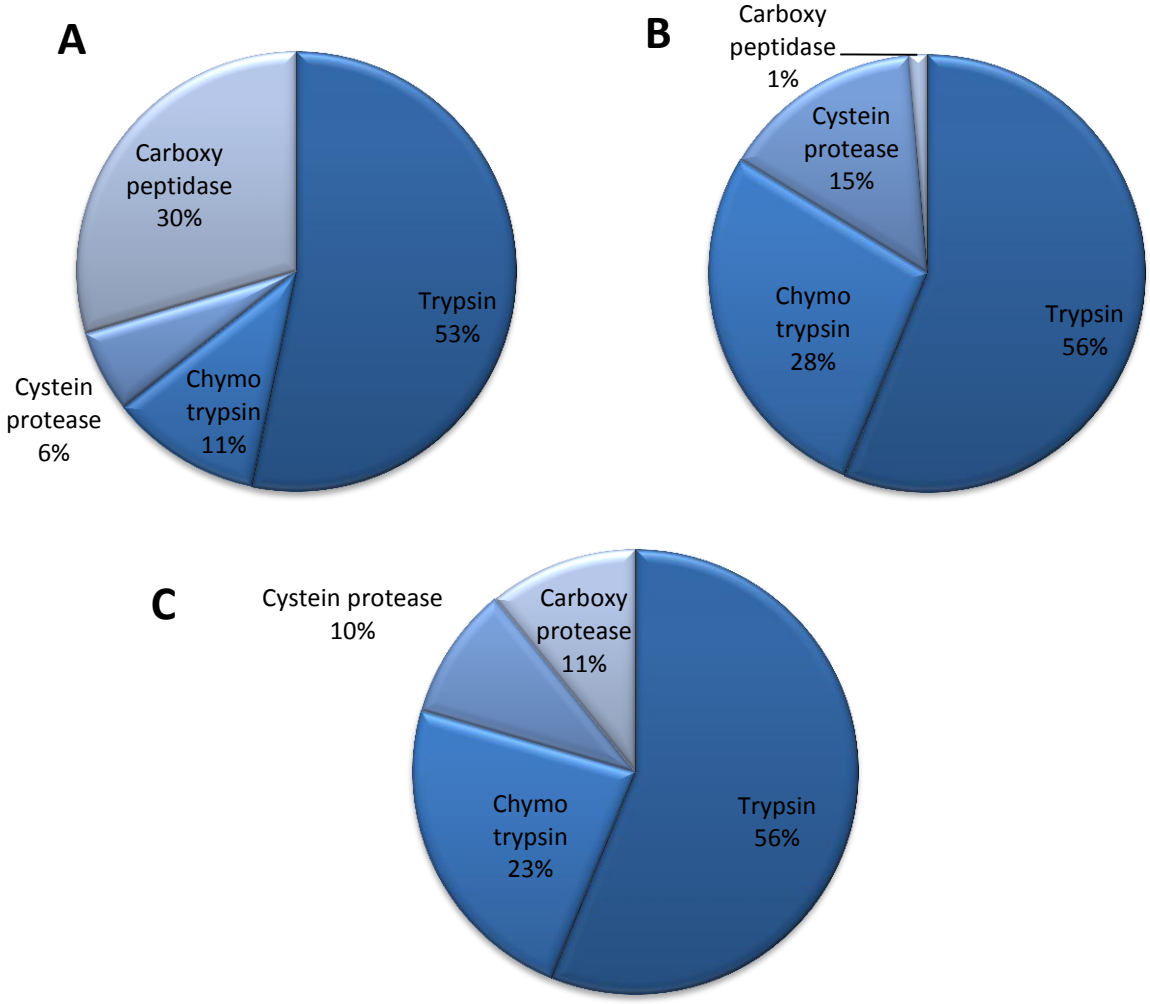


Fig. 2.4 Relative midgut proteolytic activities of 4 different proteases estimates under three different feeding regimes: basic enzymatic activity of sugar-fed flies (A), highest enzymatic activity of liver-fed flies measured 4 hours ppf (B) and very low enzymatic activity of decapitated liver-fed flies 4 hours ppf (C). In all tested conditions, trypsin, in relative terms, was always the most abundant enzymatic contributor (four biological replicates of three pooled guts each).

Detailed analysis of the changes in trypsin activity (by the use of an enzyme-specific substrate) showed that the activity profile of that enzyme was similar to the total proteolytic activity profile observed in liver-fed flies (shown in Fig. 2.2). Those results also confirmed the outcome of the previous experiment concerning trypsin as being the main proteolytic contributor that always represented half of the total proteolytic activity of all measured proteases (Fig. 2.5).

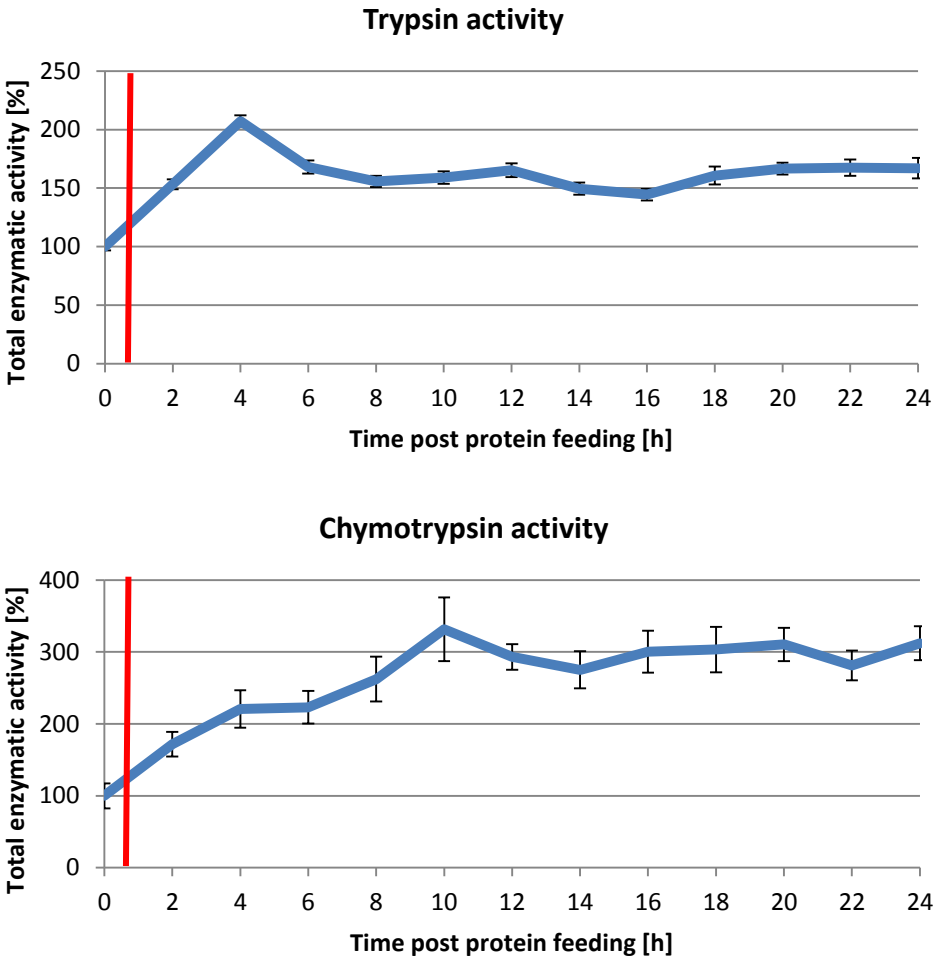


Fig. 2.5 Trypsin and chymotrypsin activity changes observed in liver-fed female flies measured up to 24 hours ppf. Both enzymes show an immediate increase in their activity in response to the protein meal intake. Nevertheless both enzymes display different activity profiles within the first 24 hours ppf. Red lines indicate the end of feeding moment (four biological replicates of five pooled midguts each); mean ± *SD*.

Similar analysis of detailed post-liver feeding changes in the chymotrypsin action revealed another activity profile, different from trypsin. That enzyme raised its activity constantly, up

to ten hours ppf and afterwards proceeded on a peak level of 300% of the basic activity observed in sugar-fed flies; and that proceeded minimally up to 24 hours ppf (Fig. 2.5). Those results also confirmed the observation of the previous experiment, which indicated a significant increase in chymotrypsin activity in liver-fed flies.

2.3.4 Trypsin gene expression analysis

The analysis of the transcriptome revealed several nucleotide sequences that could be potential trypsin cDNA's. Finally, four of them were revealed as encoding trypsin: **HAHN.FLY.9824.C1**; **HAHN.FLY.5249.C1**; **HAHN.FLY.10868.C3** and **HAHN.FLY.124.C2** (Supp. fig. 2.5). Due to the occurrence of numerous mistakes in the four reported sequences, only one sequence (**HAHN.FLY.10868.C3**) resulted in a long open reading frame of 255 amino acids (Fig. 2.6). As expected, this identified trypsin sequence contained all trypsin conserved amino acid regions, as well as some specific amino acid motifs that determined the sequence as early trypsin.

>HAHN.FLY.10868.C3

MLKFVILLSVVACAFGAVVPEGMLPQLDGRIVGGEATTISSIPWQISLQRNGGHSCGGSIYSSNIIVTAAHC

LQYVSTSVLSVRAGSSYWNTGGVVSKVAAFKNHEGYNARTMVNDIAVIRLASSLTFSSTIKTIELATVAPA

NGASASVSGWGTTSYGGSIPTQLRYVDVKIVSESSCASSSYGYGSEIKPTMICAYTVDKDACQGDSGGPLV

SGGRLVGVVSWGYGCAYTNYPGVYADVAVLRSWVVNAANSV

Fig. 2.6 Translated amino acid sequence of the **HAHN.FLY.10868.C3** gene transcript that is determined to be an early trypsin gene. Conserved amino acid regions characteristic for all trypsin sequences are marked in black, whereas the early trypsin specific amino acid motifs are marked in gray.

The other three identified nucleotide sequences resulted in a very short open reading frame. However, general trypsin and specific early and late trypsin amino acid motifs were found. Their specific amino acid motifs analysis, established three early trypsin genes: **HAHN.FLY.9824.C1; HAHN.FLY.5249.C1** and **HAHN.FLY.10868.C3** and one late trypsin gene: **HAHN.FLY.124.C2** (data not shown).

The nucleotide sequence quality of all found trypsin gene transcripts was sufficient to design specific primer pairs for each trypsin gene and to measure their expression after liver feeding by qRT-QPCR (Fig. 2.7).

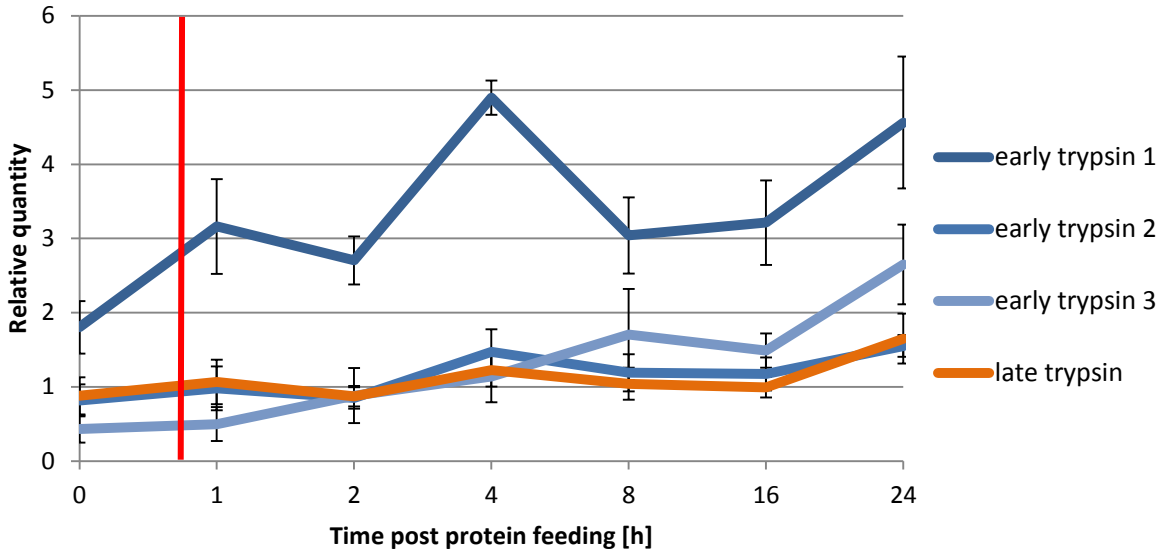


Fig. 2.7 Expression patter of different trypsin genes of *S. crassipalpis* post protein feeding. All genes show an increase in their expression level after protein meal intake. The most dominant early trypsin 1 refers to **HAHN.FLY.10868.C3**, early trypsin 2 and 3 refer to **HAHN.FLY.9824.C1** and **HAHN.FLY.5249.C1**; late trypsin refers to and **HAHN.FLY.124.C2** feeding. Red line indicates the end of feeding moment (four biological replicates of five pooled midguts each); mean ± *SD*.

Early trypsin 1 (**HAHN.FLY.108686.C3**) demonstrated the highest activity level and its transcript level started to increase after liver feeding. Similar to the results of the enzymatic proteolytic assay (Fig. 2.2), the moment of the highest activity was measured at 4 hours ppf. Early trypsin 2 and 3 (**HAHN.FLY.9824.C1** and **HAHN.FLY.5249.C1**) were much less abundant compared to the previous one. A basal level of the mRNA encoding early trypsin 2 measured at 0 hour ppf was very low but its expression was constantly rising over time.

2.3.5 Central regulation of the midgut digestion

The protein meal seemingly elicited the increase in digestive activity. In order to find out if that process was regulated locally or centrally, flies were first attracted and fed with cow liver for 45 minutes, to deliver the exogenous stimulus needed to increase digestion. By simple (mechanical) strictly time controlled decapitation of the liver fed flies, it was observed that the 'head' is needed for a minimum of 4 hours ppf in order to assure complete

protein digestion. Compared to the proteolytic activity measured in intact liver-fed insects, flies decapitated within 1 hour ppf showed only a low, not increasing level of protease activity in their midgut ($p_{2\text{-}10h}$=0.00014) (Fig. 2.8A). The measured enzymatic activity in the midgut of those decapitated flies, dropped below the basal activity level of the enzymes secreted by sugar-fed flies.

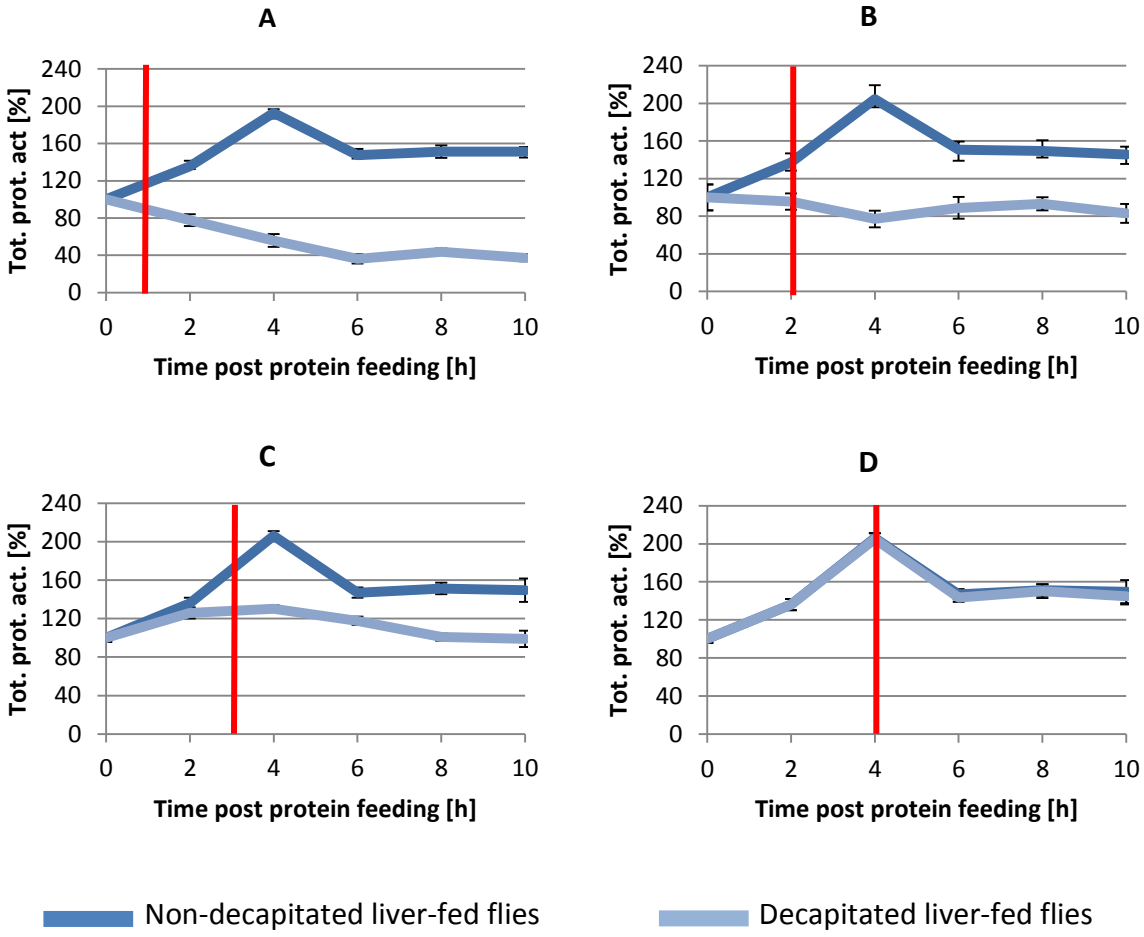


Fig. 2.8 Midgut proteolytic activity of intact liver-fed flies compared to liver-fed flies decapitated at 1 hour (A), 2 hours (B), 3 hours (C) and 4 hours (D) time ppf. Decapitation after 4 hours ppf is no longer influencing proteolytic digestive activity. Red lines indicate decapitation moments (four biological replicates of three pooled midguts each); mean ± *SD*.

Also the enzymatic activity in flies decapitated after 2 hours ppf did not increase, but oscillated around the starting basal activity level (p_{2h}=0.00029; $p_{4\text{-}10h}$=0.000127) (Fig. 2.8B). The first increase in measured proteolytic digestion was observed in the group of flies decapitated after 3 hours ppf. Nevertheless, there were significant differences between that

group and the positive control group (p_{4h}=0.000271; p_{6h}=0.236631; p_{8h}=000129; p_{10h}=0.000139).

Decapitation after 4 hours ppf, or at later time points ppf, could no longer prevent the full increase in digestive activity (fig. 2.8D) and flies decapitated after that time-point showed a midgut total proteolytic activity profile similar to that in the liver-fed positive control group (p_{4h}=0.95732; p_{6h}=0.999852; p_{8h}=1.000000; p_{10h}=0.998643).

2.3.6 Extracts of corpora cardiaca *as effective elicitors of digestion*

Based on the fact that CC represents an organ containing several brain-derived neuropeptides involved in feeding/digestion regulation, flies were fed with liver and immediately decapitated (to remove their own 'head factor') and subsequently injected with a CC peptide extract, which was derived from flies only fed with sugar (to avoid any post protein feeding induced release of the potential digestion regulating factor), in order to rescue the digestion process.

Due to the fact that every extraction/purification procedure causes the loss of some biological material, we first tested the dose response effects by injecting a different number of CC equivalents into the decapitated liver-fed flies, in order to determine the amount of the peptidergic extract causing response as seen in the intact liver-fed flies (Fig. 2.9).

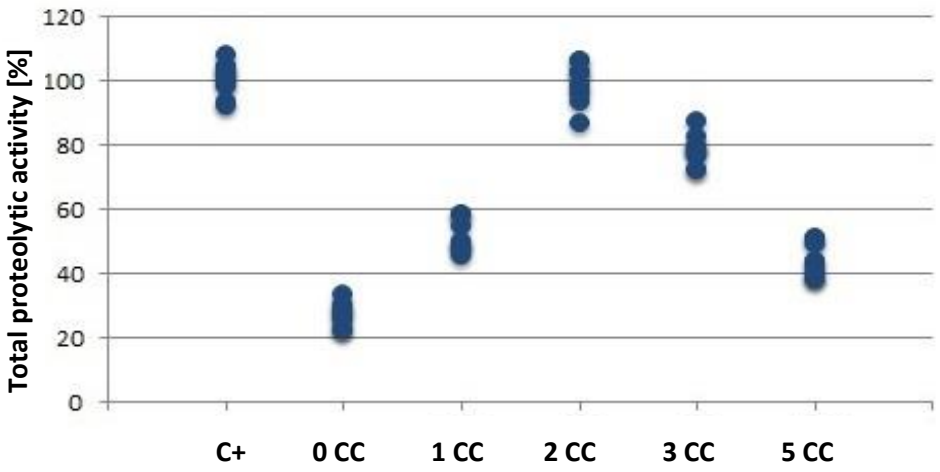


Fig. 2.9 The dose response effect of different number of the CC extract equivalents injected into liver-primed decapitated female flies. Midgut proteolytic activity was measured at 4 hours ppf. Peptidergic extract obtained from two CC equivalents results in the most similar proteolytic activity as observed in intact liver-fed flies. C+ refers to proteolytic activity measured in the positive control midguts of non-decapitated liver-fed females (*n= 10 individual organisms).*

Injection of the peptidergic extract obtained from one CC equivalent was clearly sufficient to enhance the midgut proteolytic activity in decapitated liver-fed females. Injection of two CC equivalents elicited a much higher enzymatic activity in the midguts of all tested flies and it almost reached the same level of non-decapitated liver-fed controls (p_2=0,94682). An injection of three equivalents was still effective, although less pronounced. Last tested dose of five equivalents was again less effective and no more pronounced than that of two CC equivalents. Student T-test confirmed differences between zero CC injected dose versus one, two, three and five CC equivalents ($p_{1,2,3,5}$= 0).

We also set out rescue experiments, in which CC extracts were prepared from flies at different times post liver feeding. By these rescue experiments, injection of two CC equivalents into decapitated liver-fed flies confirmed the need for (neuro)peptidergic regulation of the digestive activity in flies. There was a significant difference between decapitated CC extract injected liver-fed flies and Ringer injected decapitated liver-fed flies (p_{2h}=0.00119; $p_{4\text{-}6h}$=0.000143). Decapitated flies injected with CC derived from sugar-fed flies displayed a normal proteolytic activity profile compared to intact liver-fed insects (p_{0h}= 1.00000; p_{2h}=0.97417; p_{4h}=0.93304; p_{6h}=0.64916) (Fig.2.10A). The same positive results were observed using CC extracts collected from flies 1 and 2 hours ppf (Fig. 2.9B and Fig. 2.9 C respectively). Injection of the peptidergic extract prepared from CC of flies dissected after 3 hours ppf gave similar result as flies decapitated after 3 hours ppf. The measured enzymatic activity again oscillated around the starting level for 4 hours (Fig. 2.10D) and then suddenly decreased to the level of decapitated liver-fed flies. These pooled gut samples were also characterized by a huge variation, especially at 4 hours ppf when some single replicas reached a very high digestive activity, similar to the positive control of non-decapitated liver-fed flies, whereas the other replicates presented very low proteolytic activity, which was similar to the negative control. CC extracts obtained from the flies which were liver fed for 5 and 20 hours were no longer able to restore the physiological level of digestion as observed in the non-decapitated liver-fed control flies (Fig. 2.10E and Fig. 2.10F respectively). The proteolytic activity of flies injected with those extracts was similar to the one observed in the negative control of decapitated liver-fed flies (p_{0h}=1.00000; p_{2h}=0.85902; $p_{4\text{-}6h}$=1.00000).

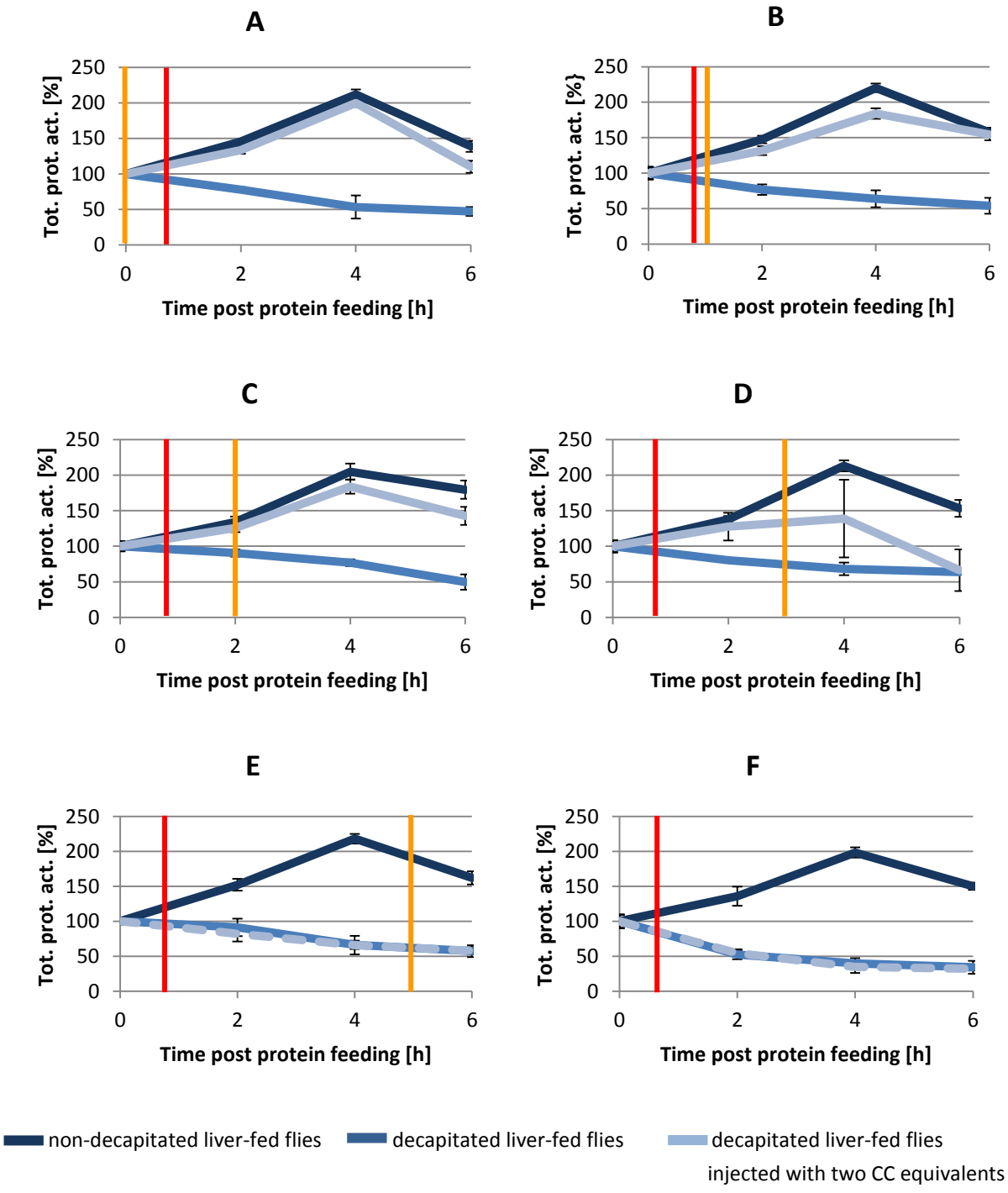


Fig. 2.10 Midgut proteolytic activity of intact liver-fed flies, decapitated liver-fed flies and decapitated liver-fed injected with two CC equivalents of sugar fed females (A), liver fed flies 1 hour ppf (B), 2 hours ppf (C), 3 hours ppf (D), 5 hours ppf (E) and 20 hours ppf (F). Red lines indicate the end of feeding, decapitation and injection moments, whereas orange lines indicate moments of the CC collection to prepare the peptidergic extracts (from the CC donor group of flies) (four biological replicates of three pooled midguts each); mean *± SD*.

2.4 Discussion

Physiological regulation of digestion is a very complex process, controlled by multiple factors on different levels. It is evident that in all anautogenous insects, the protein meal stimulates midgut to much more intensive synthesis of digestive enzymes (Borovsky *et al.*, 1996). These significant changes in enzymatic (proteolytic) activity indicate that the used anautogenous flesh fly species, similar to mosquitoes, represent a group of batch digesters. Feeding with the complex protein meal (liver) doubles the proteolytic activity in the gut homogenate. Although the increase is considerable, the rise is not as spectacular as it is observed in mosquitoes, in which the blood meal elicits the activity of trypsin and chymotrypsin to increase about ten folds (Okuda *et al.*, 2005).

It is possible that the changes in the proteolytic activity between sugar and liver-fed flies would have been larger, if a solution containing only secreted enzymes was used, instead of the midgut homogenate. Homogenization of the sample causes the release of all enzymes (even the stored one) into the solution; in contrast to the secretion assay that demonstrates much more accurate changes in gut activity. Unfortunately, it was not possible to perform the midgut secretion assay in our model organism, as the midgut preparation could not be freed from the coagulated gut content. Nevertheless, also other previous researchers reported the successful use of a midgut homogenate in a related species, *Neobellieria* (*Sarcophaga*) *bullata,* to study the proteolytic activity changes of post liver feeding (Bylemans *et al*., 1994).

Independent of the nutritional state, the main protease (constituting a little bit more than 50% of all proteolytic enzymes) present in the midgut of all Diptera, including *Sarcophaga crassipalpis*, is trypsin (Borovsky *et al.*, 1996). Its activity profile resembles in shape the total proteolytic activity curve, showing its strong contribution in overall protein digestion. Next to trypsin, chymotrypsin represents the second highest proteolytic activity measured in the midgut of liver-fed flies. Interesting is also its activity profile which seems to increase significantly after liver feeding having an activity profile which is different from that of trypsin. It is worth considering that the increase of the relative chymotrypsin activity is observed in intact protein-fed, as well as in decapitated protein-fed flies. Its proportional activity increases 2-2.5 folds, in comparison with the basal chymotrypsin activity of sugar-fed

flies. It seems that its appearance is induced by the ingestion of the protein meal, as is the case for mosquito late trypsin. This explains and also confirms its 'late' appearance which is evoked by the intake of protein (Borovsky *et al.*, 1996). Some recent literature referring to proteolytic digestive enzymes in insects, by using a phylogenetic sequence analysis, shows that mosquito late trypsin clusters within the chymotrypsin subgroup (Spit *et al.*, 2014). It is also suggested that late trypsin can derive from a chymotrypsin ancestor (Marshall *et al.*, 2008).

These protein meal induced changes in enzyme activities, make the herein reported observation for *Sarcophaga crassipalpis* in agreement with the changes in proportional specific enzyme activities in blood sucking anautogenous mosquitoes (Muller *et al.*, 1993;Lu *et al.*, 2006).

Noteworthy, using qRT-PCR, our data indicates at least a partial (liver meal induced) transcriptional regulation of the trypsin activity in the midgut. This result is seemingly in conflict with earlier findings regarding trypsin regulation in the anautogenous flesh fly, *Neobellieria bullata* (Borovsky *et al*., 1996).

Anautogenous flies such as *Sarcophaga crassipalpis*, do not respond to a free amino acid meal, which is devoid of protein. This data confirms the findings in another anautogenous insect, *Aedes aegypti* (Gulia-Nuss *et al.*, 2011). Statistical analysis also proves that there is no significant difference in proteolytic activity between sugar-fed and free amino acid-fed flies. This strongly indicates that only a protein meal affects gut protein digestion.

The results of our time-controlled decapitation experiments, clearly suggest that the process of digestion is stimulated by a head factor. Similar to our observations, early (1 hour post feeding) decapitation in mosquitoes also prevents blood meal digestion (Gulia-Nuss *et al.*, 2011). Altogether, this data suggests that the release of the digestion stimulating factor begins after protein consumption. In *Sarcophaga crassipalpis*, the massive release of this factor occurs between 3 and 4 hours post feeding. Nevertheless, once the process is fully started, it is no longer sensitive to any disruption. Late decapitation after 4 or more hours post liver feeding, has no longer any effect on the measured level of the enzymatic activity.

This digestion stimulating factor is most probably peptidic in nature. Injection of the CC peptidergic extract, into the decapitated liver-primed flies, allows a rescue of the digestion process in these insects, provided that the CC was taken from flies in which the peptide release event did not yet occur. Extracts collected from sugar-fed or from 'early' liver-fed flies restores proteolytic activity in decapitated insects, even up to the physiological level. A peptide mixture isolated from the CC of flies after 5 hours post feeding or later, no longer contains the digestion stimulating factor (it is probably already released into the hemolymph). The results of 'active/early' and 'non-active/late' CC extracts confirm our previous observations of 'early' and 'late' decapitation.

The digestive process in all experiments is synchronized by the moment of accessibility of liver. Flies attracted by the smell of meat immediately start eating. Both our decapitation and rescue experiments that use extracts of CC derived from flies at different time points post liver feeding, strongly suggest either a complete release or *in situ* inactivation of the CC factor responsible for stimulating digestive activity in the anautogenous flesh fly.

CHAPTER 3.

Neuropeptidergic regulation of digestion in the anautogenous flesh fly, *Sarcophaga crassipalpis.* Adipokinetic hormone as a digestion regulator. ♯

♯ Parts of this chapter are published in: Bil,M.; Broeckx,V.; Landuyt,B.; Huybrechts,R. (2014) Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, *Sarcophaga crassipalpis*. General and Comparative Endocrynology 208, 49-56.

3.1 Introduction

Neuropeptides are small messenger molecules that are widespread among the kingdom Animalia. They are involved in the control of a variety of processes, such as behavior, metamorphosis, digestion, reproduction, etc. They originate from neuro-endocrine cells, and are derived from large precursor molecules (prepropeptides) that are subsequently cleaved and modified to become biologically active peptides. Peptidergic neurons can be divided into two groups: interneurons that mainly modulate actions within the central nervous system, and neurosecretory neurons that produce neurohomones and store them in the neurohemal organs, which are in direct contact with the hemolymph. In insects, the main neurohemal organ is the retrocerebral complex, which consists of *corpora allata* and *corpora cardiaca* (CC) (Rahman *et al.*, 2013).

In *Sarcophaga crassipalpis*, the CC is located behind the brain, in the very upper part of the thorax. It is situated close to the joint of esophagus and proventriculus (Fig. 3.1) (oral message and personal demonstration by Dr. Heather Marco; South Africa).

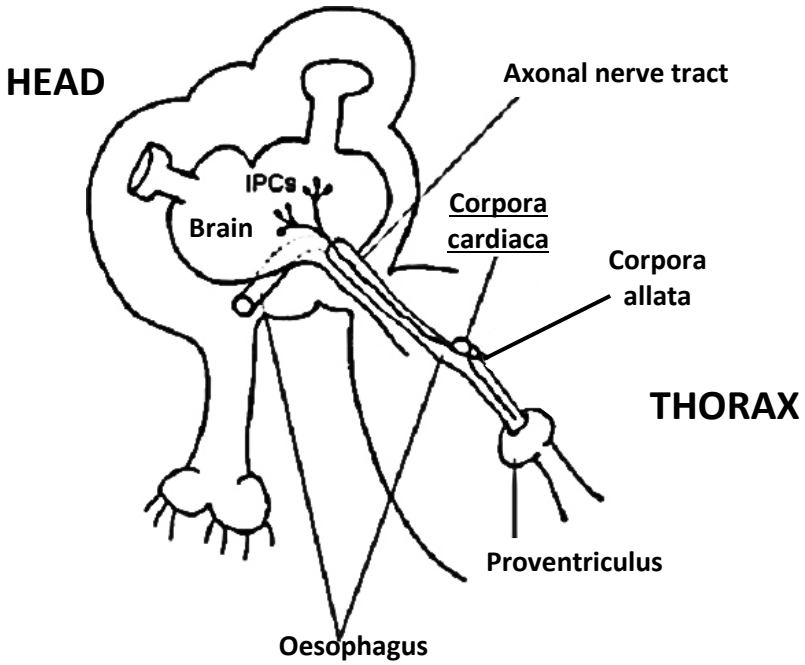


Fig. 3.1 Localization of the retrocerebral complex (*corpora cardiaca-corpora allata*) in the flesh fly body cavity. *Corpora cardiaca* is situated in the upper part of the thorax, attached to the oesophagus, close to the proventriculus. Abbreviations: IPCs, insulin producing cells. Image credits: researchgate.net

There are some published analyses of the neuropeptides that can be found in the retrocerebral complex of some fly species. The CC of the blowfly, *Lucilia cuprina,* contains two forms of Yamides (predicted homologs to an amidated eclosion hormone (Zoephel *et al.*, 2012)); three forms of the short neuropeptide F and its propeptide (regulation of digestion in the cockroach, *P. americana* (Mikani *et al.*, 2012)); pyrokinin (modulation of the feeding behavior in the fruit fly, *D. melanogaster* (Baker *et al.*, 2007)), myosuppressin (inhibition of the gut contraction and food intake in the leafworm, *S. littoralis* (Vilaplana *et al.*, 2008)) and corazonin (regulation of the crop contraction and food transfer in the blowfly, *P. terranvae* (Cantera *et al.*, 1994)) (Rahman *et al.*, 2013). The same neuropeptids, enriched with an additional intermediate form of adipokinetic hormone (AKH) (stimulation of digestive enzymes in the firebug, *P. apterus* (Kodrik *et al.*, 2012), are found in the CC of the flesh fly, *Neobellieria bullata* (Rahman *et al.*, 2013).

In the previous chapter, we investigated the proteolytic digestive activity and its changes in the anautogenous flesh fly, *S. crassipalpis*, under different dietary conditions. It was showed that this process, initiated by the ingestion of a protein meal, was regulated centrally and,

once started, preceded up to the critical level around 4 hours ppf, could no longer be interrupted by decapitation. Importantly, the use of a peptide extract of CC collected from flies prior to the critical moment of 4 hours ppf, was able to fully rescue the proteolytic digestion in decapitated liver-fed flies.

Based on that information, using mass spectrometry tools and an approach of differential peptidomics, we characterized the peptides that were present in the CC of *S. crassipalpis* either before or after the ingestion of a protein meal. We also examined the quantitative changes of all those peptides of the CC, following the protein feeding. It resulted in the pinpointing of AKH as being a proteolytic enzyme stimulator. *In vivo* injection of the AKH peptide into sugar and liver-fed flies confirmed its role in the regulation of digestion. To ensure that the quantitative differential peptidomics search had not missed any important digestion regulating peptide, all other identified CC peptides were tested for *in vivo* activity and all of them gave negative results.

3.2 Material and methods

3.2.1 Insect rearing

Flies used for experiments were reared as described in chapter 2, paragraph 2.2.1.

3.2.2 Peptide extraction

Peptides used for mass spectrometry analyses were extracted as described in chapter 2, paragraph 2.2.9.

3.2.3 MALDI-TOF mass spectrometry

The qualitative mass spectrometry analysis of the neuropeptides that are present in the *corpora cardiaca* was performed on a matrix assisted laser desorption/ionization time-of-flight UltraFlex II instrument (MALDI-TOF, Brucker Daltonics, Germany). The samples were dissolved in 70% acetonitrile containing 0.1% trifluoroacetic acid and spotted on a stainless steel MALDI plate in a volume of 0,5 µl and covered with 0,5 µl of a α-cyano-4-hydroxycinnamic acid matrix saturated in 50% acetone, 0,1% trifluoroacetic acid. Analytes

were measured in positive ion mode, reflectron mode and an m/z range from 700 to 2000. The instrument was calibrated using a peptide calibrant containing nine standard peptides: angiotensin II, angiotensin I, substance P, bombesin, adrenocorticotropic hormone clip 1-17, adrenocorticotropic hormone clip 18-39, somatostatin 28, bradykinin fragment 1-7 and renin substrate tetradecapeptide porcin (Peptide Calibration Standard II, Bruker Daltonics). Due to the measurement specificity, only positive singly charged molecules were detected.

3.2.4 Quantitative ESI-MS analysis

The differential electrospray ionization mass spectrometry (ESI-MS) experiment was performed on a Q Exactive orbitrap mass spectrometer (Thermo Fisher, Germany). The mass spectrometer was coupled to an Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) instrument (Thermo Fisher, Germany). The UHPLC system was equipped with a 2 µm particle diameter, 100 Å pore size Easy Spray Pepmap RSLC C18 column, 50 µm x 15 cm (Thermo Fisher, Germany). Before sample separation on the analytical column, the lyophilized sample was resuspended in 10 µl of a 2% acetonitrile 0.1% formic acid solution (FA). Then, a 5 µl sample volume was injected and washed on a 3 µm, 100 Å, nanoviper, Acclaim Pepmap 100 C18 precolumn, 75 µm x 2 cm (Thermo Fisher, Germany). Sample separation was performed using a 60 min gradient of 100 % H_2O, 0.1% FA (mobile phase A) and 20 % H_2O, 80 % ACN, 0.08 % FA (mobile phase B). A linear gradient of mobile phase B in mobile phase A from 4 % to 55 % in 30 min followed by a steep increase to 90 % mobile phase B in 1 minute was applied at a flow rate of 300 nl/min. The Q Exactive mass spectrometer was operated in a data dependent mode. All mass spectra were acquired in the positive ionization mode with an m/z scan range of 400 to 1600 thomson (Th). For each precursor spectrum, up to the ten most intense ions were selected for the generation of fragmentation spectra. A resolving power of 70,000 full width at half maximum (FWHM) was used with an automatic gain control (AGC) target of 3,000,000 ions and a maximum ion injection time (IT) of 80 ms for precursor spectra. For fragmentation spectra, a resolving power of 17,000 FWHM was used with an AGC target of 200,000 ions and a maximum IT of 56 ms. Dynamic exclusion of 10 s was applied in order to avoid repeated fragmentation of the most abundant ions. A charge exclusion of 1^+, 5^+, 8^+ and $<8^+$ was applied concerning ion selection. Due to the measurement specificity, not only single but also multiple charged (positive) molecules could be detected.

For statistical reasons, samples of both conditions were prepared in four biological replicas.

3.2.5 Database searching

The Peaks studio software (Version 7, Bioinformatics solutions Inc., Waterloo, ON, Canada) workflow was used to analyze the fragmentation spectra. This software contains four modules: a module for *de novo* sequencing of MS/MS spectra, a Peaks DB search module for database driven peptide identification, a Peaks PTM search module for detection of post-translational modifications and a Peaks Spider search module designed to detect peptide mutations and perform homology search (Han et al., 2005; Ma and Lajoie, 2009; Han et al., 2011; Zhang et al., 2012). Spectra with the same mass were merged and a default quality threshold of 0.65 was applied. All spectra were searched against the Swiss-Prot database (version December 2013), with the taxonomy set to *Drosophila melanogaster*. Following search parameters were used: a precursor mass tolerance of 8 ppm using monoisotopic mass and a fragment mass tolerance of 15 mmu. The -10logP score was set for every sample to allow a maximal FDR of 5% for peptide spectrum matches. No digestion enzyme was selected. The following post-translation modifications (PTMs) were selected as variable modifications: oxidation (+15.99), acetylation (+42.01), amidation (-0.98), glycine-loss and amidation (-58.01), phosphorylation (+79.97), pyro-glu from E (-18.01), pyro-glu from glutamine (-17.03), sulfation (+79.96), sodium adduct (+21.98) with a maximum of two allowed variable PTMs per peptide.

Differential peptidomics analysis was performed using the Progenesis LC-MS software software (version 3.0, Nonlinear Dynamics; Durham, NC, USA). The .RAW data files from the QExactive were imported into the software. This software compresses the LC-MS data by modelling the elution peaks with minimal data loss (using a wavelet-based peak-modelling algorithm). The optimal LC-MS run for the alignment was automatically chosen, and the alignment of all the runs did not need further manual curation. An aggregate dataset is created from the aligned runs from which the peptide peaks are detected (two to eight times charged). The MS scan of each run for ion abundance quantification, so no MS/MS data is used for the quantification. The peptide ion intensities were normalised using the total intensities of all proteins. Afterwards, an .mgf file was created and send to an in-house mascot server using the Swissprot database with the taxonomy for *Drosophila*

melanogaster. C-terminal amidation, conversion of N-terminal glutamic acid to pyro-glutamic acid and methionine oxidation were selected as variable modifications. No digestion enzyme was specified. Peptide mass tolerance was set at ten parts per million (ppm) and fragment mass tolerance at 20 milli mass units (mmu). The identifications from mascot were imported into Progenesis. A t-test was used to identify the significant differences between the groups.

3.2.6 Peptides

For *in vivo* testing the used synthetic equivalents of AKH (pQLTFSPDWa), short neuropeptides F-1 and F-3 (AQRSPSLRLRFa and KPQRLRFa respectively), corazonin (pQTFQYSRGWTNa) and myosuppressin (TDVDHVFLRFa), all correspond in sequence to the endogenous peptides of *Drosophila melanogaster*.

The peptides were synthesized by GL Biochem Ltd. (Shanghai) and purification was performed in house using reversed-phase high performance liquid chromatography (HPLC). The purification efficiency was controlled as described by Caers (2012) and the obtained concentration was determined using bicinchoninic acid (BCA) (Stoscheck, 1990). Subsequently, the purified peptides were dried and dissolved firstly in pure DMSO (if necessary) and then further diluted in fly Ringer's solution, in order to obtain the particular concentration. Each fly received a particular dose of the peptide in a 1 µl injection applied via the second abdominal intersternite membrane.

3.2.7 Dose-response experiment

To define the most accurate dose of AKH that induces the best physiological response, flies were fed with liver for 30 minutes, decapitated and subsequently injected with different AKH doses (0 ng, 10 ng, 100 ng, 500 ng, 1 µg corresponding to 0 mM, 0.01 mM, 0.1 mM, 0.51 mM, 1,03mM) dissolved in Ringer solution containing 10% DMSO. The evoked increase in digestive activity was measured at 4 hours post protein feeding (the moment of the highest proteolytic activity). Each particular dose was tested on 10 individual flies.

3.2.8 Feeding and decapitation procedures

Used feeding regimes and the technique of fly decapitation as described in chapter 2, paragraph 2.2.4.

3.2.9 Midgut dissection

The tissue dissection procedure was the same as described in chapter 2, paragraph 2.2.2.

3.2.10 Determination of midgut proteolytic activity

Digestive proteolytic activity was measured as described in chapter 2, paragraph 2.2.5.

3.2.11 Adipokinetic hormone immunolocalisation

Immunohistochemistry staining was performed on whole mount tissue of *corpora cardiaca.* Seen the amino acids sequence identity, the primary rabbit antibodies (polyclonal) were prepared against *D. melanogaster* AKH and constituted a kind gift from Prof. Jan Veenstra (Isabel *et al.*, 2005).

For easy manipulation, CC was dissected together with a piece of gut where it was attached. Following dissection from 4 days old sugar-fed female flies, the gut-CC tissues were fixed in 0.1 M PBS solution containing 2% paraformaldehyde for 4 h. Afterwards the gut-CCs were rinsed with PBS and incubated in blocking buffer at room temperature. Afterwards the tissues were covered with the primary antibodies diluted in the blocking buffer containing 1% Triton X-100 in dilution 1:4000 and incubated overnight at 4^{o}C. Next day they were divided into two groups. The first group was rinsed with Tris buffer and incubated in the secondary antibodies GAR-biotin (goat anti rabbit secondary antibodies, Dako) diluted in Tris buffer in dilution 1:200. Afterwards they were rinsed once again with Tris buffer and incubated with streptavidin-horseradish peroxidase (Dako). After the incubation, the preparations were again rinsed with Tris and 0.1M sodium acetate buffers. Subsequently, they were colored with the Shu coloring solution (5 g nickel ammonium sulfate, 80 mg ammonium chloride, 400 mg β-D-glucose, 2 mg glucose oxidase, 100 mg diaminobenzidine tetrahydrochloride), washed and colored with eosin for tissue visualization and embedded in paraffin. The other group was rinsed with PBS buffer and incubated with the secondary fluorescent labeled antibodies GAR/FITC (fluorescein

isothiocyanate) diluted in PBS containing 3% bovine serum albumin and 1% Triton X-100 in dilution 1:2000. Afterwards the preparations were rinsed with PBS and subsequently covered with mowiol mounting medium (Sigma-Aldrich) and transparent nail polish.

3.2.12 Statistical analysis

Statistical analysis of midgut proteolytic activity between different groups of flies, as well as dose-response experiment were tested as described in chapter 2, paragraph 2.2.8.

3.3 Results

3.3.1 Profiling of the neuropeptides present in corpora cardiaca *of* Sarcophaga crassipalpis

MALDI-TOF analysis (Fig. 3.2) obtained from a pool of 50 CCs collected from sugar-fed flies, showed 12 peaks. MALDI mass spectrometry technique generates only positive, single charged ion signals. Mass spectrometry analysis and neuropeptide annotation was largely supported by information of the fully sequenced genome of the fruit fly *Drosophila melanogaster* (Adams *et al.*, 2000) and by use of the peptidomic analysis of the flesh fly *Neobelleria bullata* and the blowfly *Lucilia cuprina* (Fig. 3.2) (Rahman *et al*., 2013).

The information of those databases allowed for identification of the peptides in nine peaks. The identified peptides were: truncated form of short neuropeptides F-1 (SPSLRLRFa), short neuropeptide F-3 (KPQRLRFa) and short neuropeptide F propeptide (author declared that the substance was partially sequenced and it likely represented an orthocopy of *Drosophila* sNPF-PP-1; however the sequences was not specified (Rahman *et al.*, 2013)), twice detected (due to the sodium adduct) adipokinetic hormone (AKH) and its two intermediates (pQLTFSPDWa; pQLTFSPDWGK-OH; pQLTFSPDWGKR-OH), myosuppressin (TDVDHVFLRFa) and corazonin (pQTFQYSRGWTNa).

The peak of m/z 1457.89 was also present in the spectrum of *N. bullata*; unluckily, it still remains unknown.

The spectrum of *S. crassipalpis* CC also displayed some extra peaks; unfortunately, not all of them could be identified or were explained as the result of contamination in the sample. The extra identified peaks were pyrokinin (VPWTPSPRLa) (Verleyen *et al.*, 2006) and CAPA propeptide (SDLDSVSEGRH-OH) (Predel *et al.*, 2010).

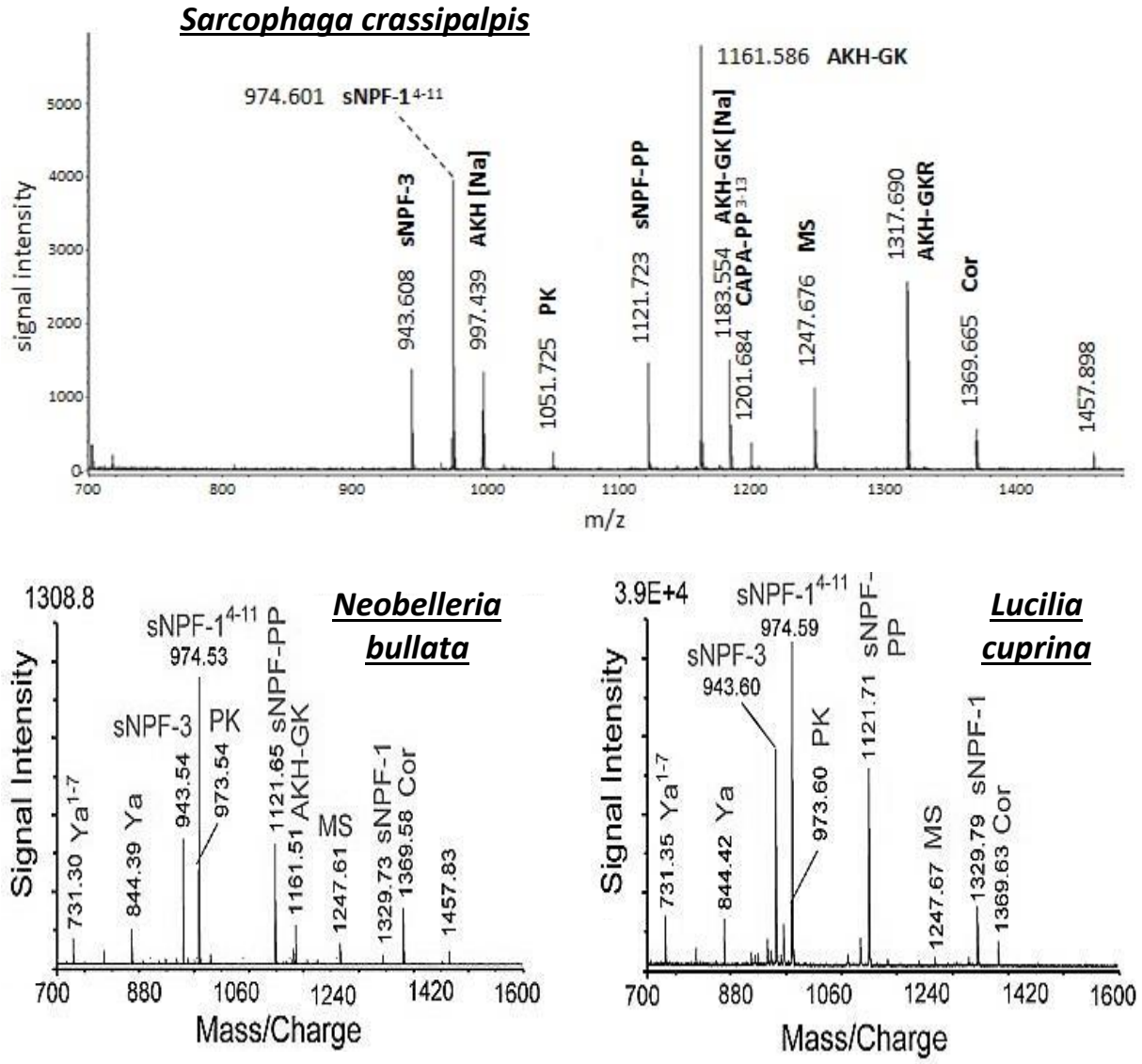


Fig. 3.2 MALDI-TOF mass spectra of *corpora cardiaca* preparations extracted from sugar-fed female flesh flies, *Sarcophaga crassipalpis*(top spectrum) and closely related flesh flies, *Neobelleria bullata* (left bottom spectrum) and blowflies, *Lucilia cuprina* (right bottom spectrum). Their comparison allows for mass-based peak identification of the *S. crassipalpis* neuropeptides. Spectra represent the results of the single, positive charged molecules detected in tested sample. Detected peptides: sNPF, short neuropeptide F; AKH, adipokinetic hormone; PK, pyrokinin; MS, myosuppressin; Cor, corazonin; CAPA-PP, CAPA propeptide. Image credits: Rahman *et al.*, 2013

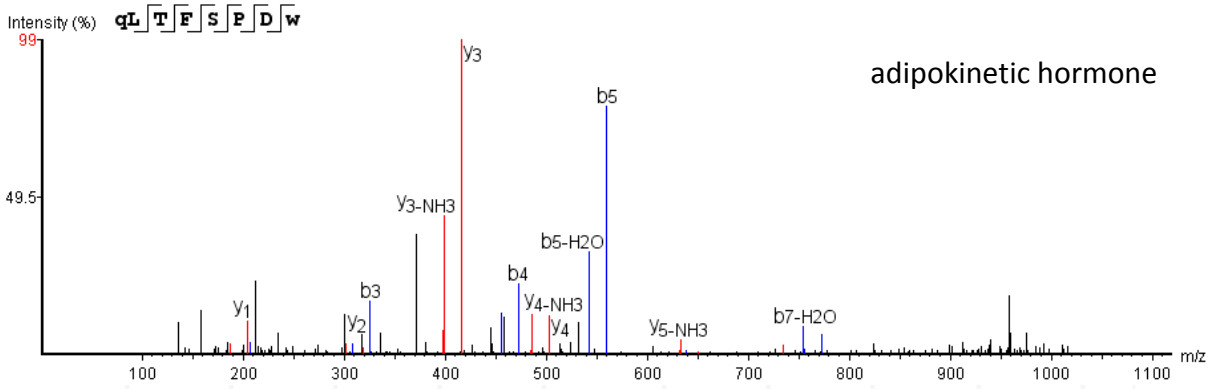
The m/z ratio spectrum was further extended up to ten kDa. Unfortunately, even using different purification methods (acidic water instead of acidic methanol and C18 purification columns), covering matrices (sinapinic acid instead of α-cyano-4-hydroxycinnamic acid) or MALDI measuring plates (hydrophobic AnchorChip MALDI-plate instead of regular stainless steel MALDI-plate), only two extra peaks of 2069.25 and 3483.12 Da with relatively low signal intensity were detected (data not shown). Even though the results were reproducible; it was not possible to identify the sequences of those particular signal peaks.

The results of the all peptides that were identified during that MALDI-TOF analysis were summarized in Table 3.1.

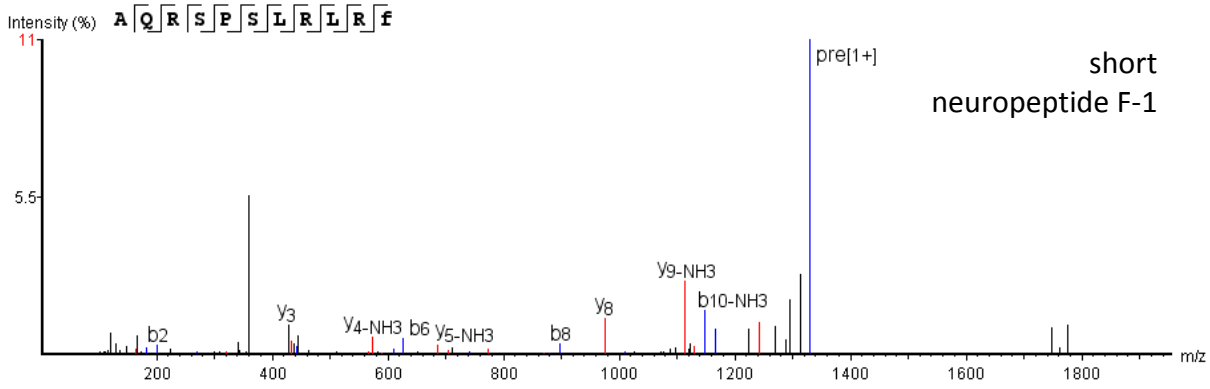
Peptide name	Sequence	Theoretical $[M+H]^+$	Measured $[M+H]^+$	Mass error [ppm]
Adipokinetic hormone				
AKH [Na]	pQLTFSPDWa	997.439	997.439	0
AKH-GK	pQLTFSPDWGK-OH	1161.551	1161.586	0.035
AKH-GK [Na]	pQLTFSPDWGK-OH	1183.540	1183.554	0.014
AKH-GKR	pQLTFSPDWGKR-OH	1317.652	1317.690	0.038
Short neuropeptide F				
sNPF-1^{4-11}	SPSLRLRFa	974.573	974.601	0.028
sNPF-3	KPQRLRFa	943.579	943.608	0.029
Pyrokinin				
PK	VPWTPSPRLa	1051.589	1051.725	0.136
CAPA propeptide				
CAPA-PP^{3-13}	SDLDSVSEGRH-OH	1201.544	1201.684	0.140
Myosupressin				
MS	TDVDHVFLRFa	1247.637	1247.676	0.039
Corazonin				
Cor	pQTFQYSRGWTNa	1369.622	1369.665	0.043

Table 3.1 List of neuropeptides found in the CC of sugar-fed fly, *S. crassipalpis*, that are identified by MALDI mass spectrometry technique.

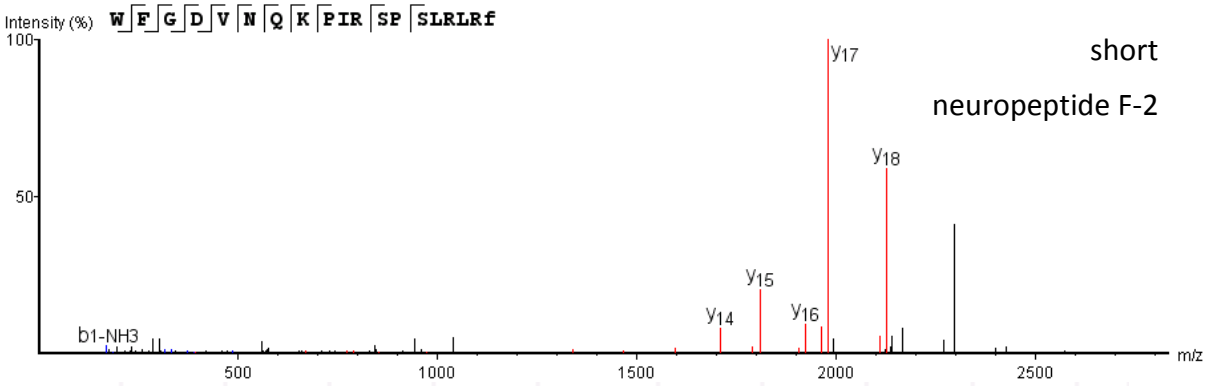
Additional peptide identification took place during the differential peptidomic experiment performed by ESI-MS. That mass spectrometry technique detected some additional peptides (not found on MALDI) of another CAPA peptide, FMRFamide and tachykinin. All peptides were fragmented to determine their amino acid sequence (Fig. 3.3).



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	112.04	94.03	95.01	56.52	Q(-17.03)					8
2	225.12	207.11	208.10	113.06	L	864.42	846.41	847.40	432.71	7
3	326.17	308.16	309.14	163.59	T	751.34	733.33	734.32	376.17	6
4	473.24	455.23	456.21	237.12	F	650.30	632.28	633.27	325.65	5
5	560.27	542.26	543.25	280.64	S	503.23	485.21	486.20	252.11	4
6	657.32	639.31	640.30	329.16	P	416.19	398.18	399.17	208.60	3
7	772.35	754.34	755.33	386.68	D	319.14	301.13	302.11	160.07	2
8					W(-.98)	204.11	186.10	187.09	102.56	1

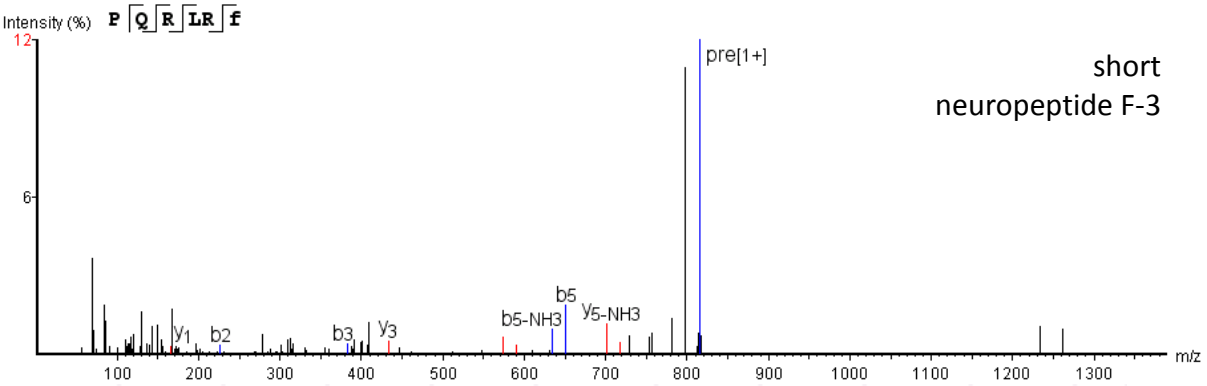


#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	72.04	54.03	55.02	36.52	A					11
2	200.10	182.09	183.08	100.55	Q	1258.75	1240.74	1241.71	629.87	10
3	356.20	338.19	339.18	178.60	R	1130.68	1112.68	1113.66	565.84	9
4	443.24	425.23	426.21	222.12	S	974.59	956.58	957.56	487.79	8
5	540.29	522.28	523.26	270.65	P	887.56	869.55	870.53	444.28	7
6	627.32	609.30	610.29	314.16	S	790.50	772.49	773.48	395.75	6
7	740.40	722.39	723.38	370.70	L	703.47	685.46	686.45	352.24	5
8	896.50	878.50	879.48	448.75	R	590.39	572.38	573.36	295.69	4
9	1009.60	991.58	992.56	505.30	L	434.28	416.28	417.26	217.64	3
10	1165.69	1147.68	1148.66	583.35	R	321.20	303.19	304.18	161.10	2
11					F(-.98)	165.10	147.09	148.08	83.05	1



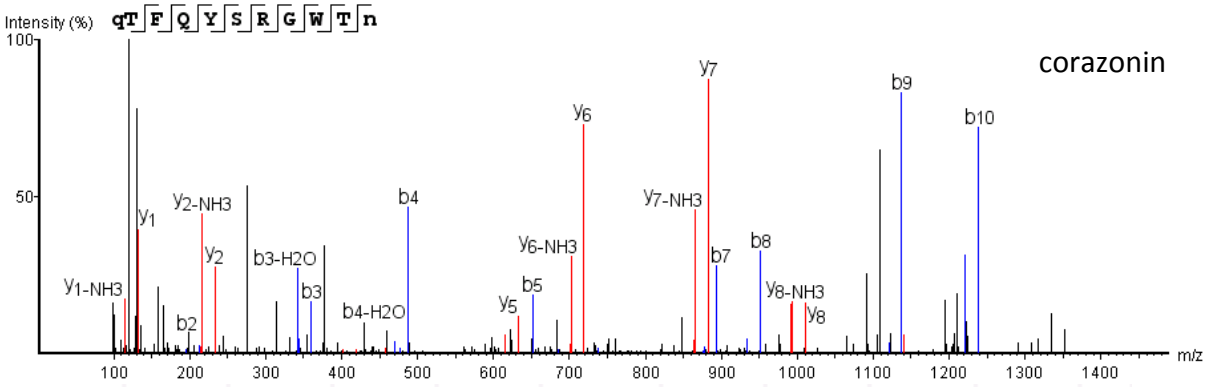
short neuropeptide F-2

#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	187.09	169.08	170.06	94.04	W					19
2	334.15	316.14	317.13	167.58	F	2129.21	2111.20	2112.19	1065.10	18
3	391.18	373.17	374.15	196.09	G	1982.13	1964.13	1965.11	991.57	17
4	506.20	488.19	489.18	253.60	D	1925.12	1907.11	1908.10	963.06	16
5	605.27	587.26	588.25	303.14	V	1810.09	1792.08	1793.07	905.55	15
6	719.32	701.30	702.29	360.16	N	1711.03	1693.01	1694.00	856.01	14
7	847.37	829.36	830.35	424.19	Q	1596.97	1578.97	1579.95	798.99	13
8	975.47	957.46	958.44	488.23	K	1468.92	1450.91	1451.90	734.96	12
9	1072.52	1054.51	1055.49	536.76	P	1340.82	1322.82	1323.80	670.91	11
10	1185.61	1167.60	1168.58	593.30	I	1243.77	1225.76	1226.75	622.39	10
11	1341.71	1323.70	1324.68	671.35	R	1130.69	1112.68	1113.66	565.85	9
12	1428.74	1410.73	1411.71	714.87	S	974.58	956.58	957.56	487.79	8
13	1525.79	1507.78	1508.76	763.40	P	887.56	869.55	870.53	444.28	7
14	1612.82	1594.81	1595.80	806.91	S	790.49	772.49	773.46	395.75	6
15	1725.91	1707.90	1708.88	863.45	L	703.47	685.46	686.45	352.24	5
16	1882.01	1864.00	1864.98	941.50	R	590.39	572.38	573.36	295.69	4
17	1995.09	1977.08	1978.07	998.05	L	434.29	416.28	417.26	217.64	3
18	2151.19	2133.18	2134.17	1076.10	R	321.20	303.19	304.18	161.10	2
19					F(-.98)	165.10	147.09	148.08	83.05	1

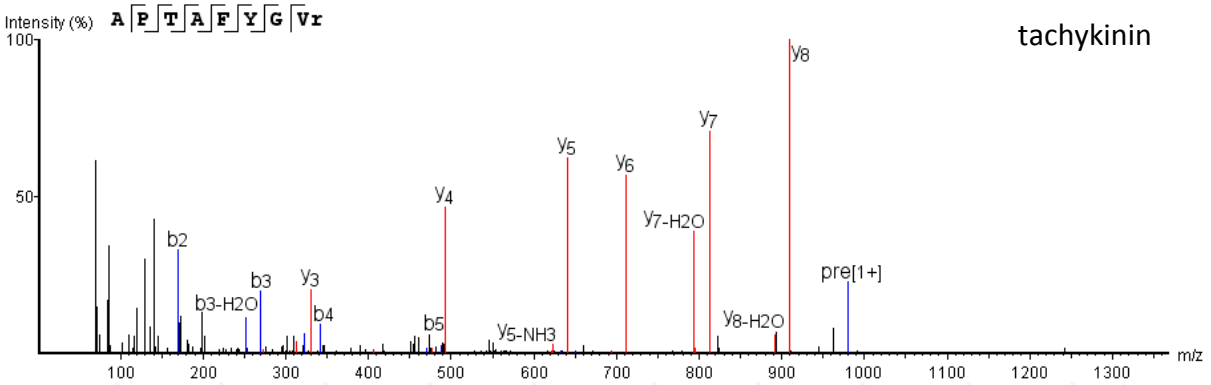


short neuropeptide F-3

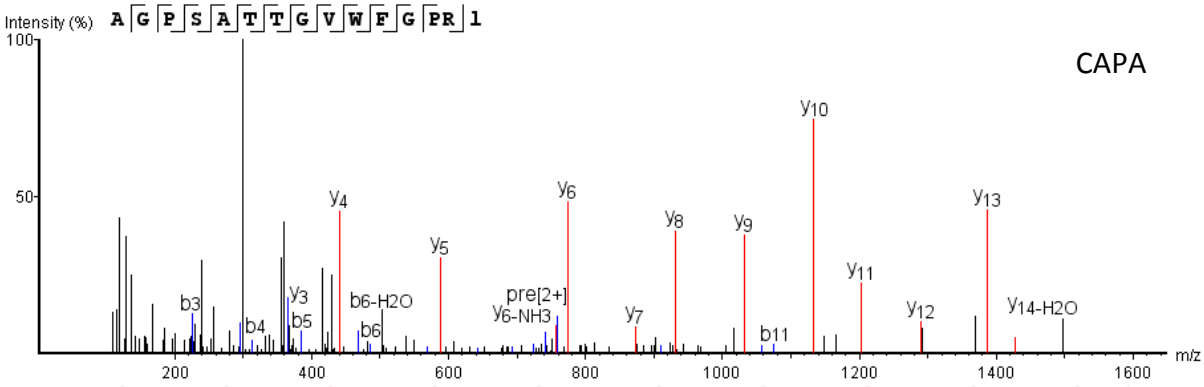
#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	98.06	80.05	81.03	49.53	P					6
2	226.12	208.11	209.09	113.56	Q	718.44	700.44	701.42	359.72	5
3	382.22	364.21	365.19	191.61	R	590.39	572.38	573.36	295.69	4
4	495.30	477.29	478.28	248.15	L	434.29	416.28	417.26	217.64	3
5	651.41	633.39	634.38	326.20	R	321.20	303.19	304.18	161.10	2
6					F(-.98)	165.10	147.09	148.08	83.05	1



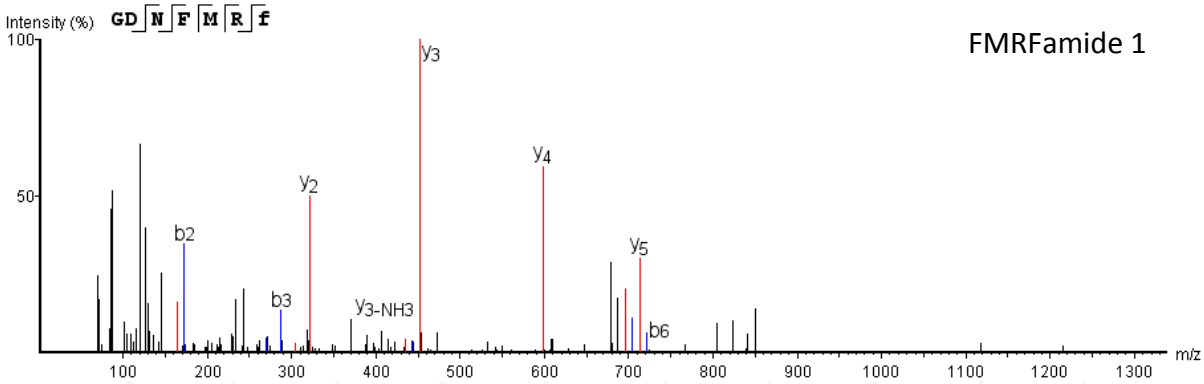
#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	112.04	94.03	95.01	56.52	Q(-17.03)					11
2	213.09	195.08	196.06	107.04	T	1258.60	1240.59	1241.57	629.80	10
3	360.15	342.14	343.15	180.58	F	1157.55	1139.54	1140.52	579.27	9
4	488.21	470.20	471.19	244.61	Q	1010.48	992.47	993.45	505.74	8
5	651.28	633.27	634.25	326.14	Y	882.42	864.41	865.39	441.71	7
6	738.32	720.30	721.28	369.65	S	719.36	701.34	702.33	360.18	6
7	894.41	876.40	877.40	447.71	R	632.33	614.32	615.30	316.66	5
8	951.43	933.44	934.41	476.23	G	476.23	458.22	459.20	238.61	4
9	1137.51	1119.50	1120.49	569.26	W	419.20	401.19	402.18	210.10	3
10	1238.56	1220.54	1221.54	619.78	T	233.12	215.11	216.10	117.06	2
11					N(-.98)	132.08	114.07	115.05	66.54	1



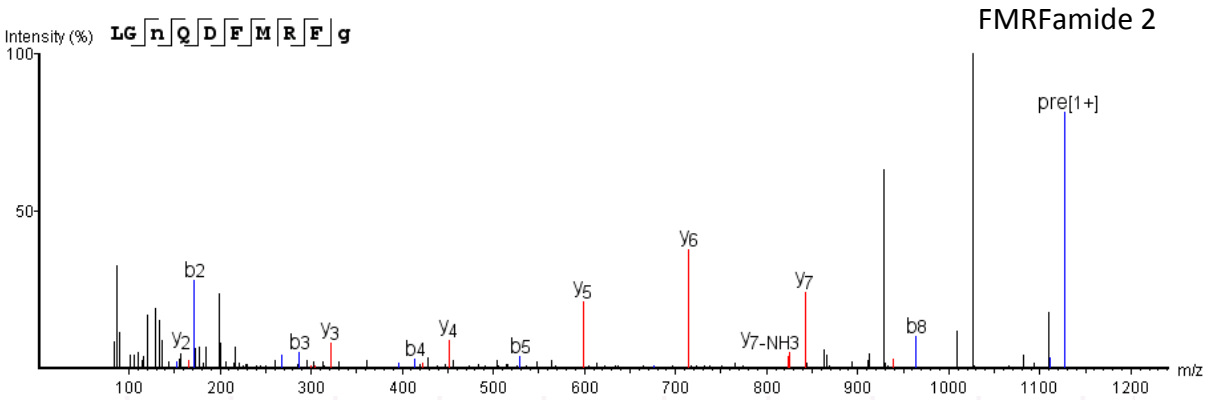
#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	72.04	54.03	55.02	36.52	A					9
2	169.10	151.09	152.07	85.05	P	909.49	891.48	892.47	455.25	8
3	270.14	252.13	253.12	135.57	T	812.44	794.42	795.42	406.72	7
4	341.18	323.17	324.16	171.09	A	711.39	693.38	694.36	356.20	6
5	488.25	470.24	471.22	244.63	F	640.36	622.35	623.33	320.68	5
6	651.30	633.30	634.29	326.16	Y	493.29	475.28	476.25	247.14	4
7	708.34	690.33	691.31	354.67	G	330.22	312.21	313.19	165.61	3
8	807.40	789.39	790.38	404.20	V	273.20	255.19	256.18	137.10	2
9					R(-.98)	174.13	156.12	157.11	87.57	1



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	72.04	54.03	55.02	36.52	A					15
2	129.07	111.06	112.04	65.03	G	1444.77	1426.77	1427.74	722.88	14
3	226.12	208.11	209.09	113.56	P	1387.75	1369.74	1370.72	694.37	13
4	313.15	295.14	296.12	157.08	S	1290.69	1272.68	1273.67	645.85	12
5	384.19	366.18	367.16	192.59	A	1203.67	1185.65	1186.64	602.33	11
6	485.23	467.22	468.21	243.12	T	1132.62	1114.62	1115.60	566.81	10
7	586.28	568.27	569.26	293.64	T	1031.58	1013.57	1014.55	516.29	9
8	643.31	625.29	626.28	322.15	G	930.53	912.52	913.50	465.77	8
9	742.37	724.36	725.35	371.69	V	873.51	855.50	856.48	437.25	7
10	928.45	910.44	911.43	464.73	W	774.44	756.43	757.41	387.72	6
11	1075.52	1057.49	1058.49	538.26	F	588.36	570.35	571.33	294.68	5
12	1132.54	1114.53	1115.52	566.77	G	441.29	423.28	424.27	221.15	4
13	1229.60	1211.58	1212.57	615.30	P	384.27	366.26	367.24	192.64	3
14	1385.70	1367.69	1368.67	693.34	R	287.22	269.21	270.19	144.11	2
15					L(-.98)	131.12	113.11	114.09	66.06	1



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	58.03	40.02	41.00	29.51	G					7
2	173.06	155.05	156.03	87.03	D	828.38	810.37	811.36	414.69	6
3	287.10	269.09	270.07	144.05	N	713.35	695.34	696.33	357.18	5
4	434.17	416.16	417.14	217.58	F	599.31	581.30	582.29	300.16	4
5	565.21	547.20	548.18	283.10	M	452.24	434.23	435.22	226.62	3
6	721.31	703.30	704.28	361.15	R	321.20	303.19	304.18	161.10	2
7					F(-.98)	165.10	147.09	148.08	83.05	1



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	114.09	96.08	97.06	57.55	L					10
2	171.11	153.10	154.09	86.06	G	1014.45	996.44	997.42	507.72	9
3	286.14	268.13	269.11	143.57	N(+.98)	957.42	939.41	940.40	479.21	8
4	414.19	396.19	397.17	207.60	Q	842.40	824.39	825.37	421.70	7
5	529.22	511.22	512.20	265.11	D	714.34	696.33	697.31	357.67	6
6	676.29	658.28	659.27	338.65	F	599.31	581.30	582.29	300.16	5
7	807.33	789.32	790.31	404.17	M	452.24	434.23	435.22	226.62	4
8	963.43	945.43	946.41	482.22	R	321.20	303.19	304.17	161.10	3
9	1110.51	1092.49	1093.48	555.75	F	165.10	147.09	148.08	83.05	2
10					G(-58.01)	18.03	0.02	1.01	9.52	1

Fig. 3.3 Fragmentation spectra of adipokinetic hormone, short neuropeptide F-1, F-2 and F-3, corazonin, tachykinin, CAPA, FMRFamide 1 and 2 derived from the *corpora cardiaca* peptidomic analysis of the anautogenous flesh, *S. crassipalpis*.

3.3.2 Differential peptidomics

The quantitative differential analysis of the peptides present in CC was performed by comparing the *corpora cardiaca* neuropeptides of flies in the different digestion stages. In the first condition, samples contained a pool of 50 CCs from sugar-fed flies. In accord with the experiments of the previous chapter (paragraph 2.3.6), this extract was fully able to stimulate midgut digestive activity in decapitated liver-fed flies. That 'active' extract was compared with the other pool of 50 CCs dissected from flies after 5 hours ppf. That peptidergic extract was no longer stimulating digestion in decapitated liver-primed insects.

ESI-MS technique can generate multiply charged ions. Similar to MALDI-TOF results, all peptides were found in several forms of different intermediates (different stages of the post-translational processing of the propeptides) or different post-translational modifications (final processing of the active peptides that protects these peptides from

the exopeptidases) that led to the creation of the biologically active/ready to be released forms of all the peptides.

Compared to sugar-fed flies, the amount of every particular peptide decreased after liver feeding. Very big variation was observed between biological replicates in both tested conditions (Supp. fig. 3.1). Nevertheless, there was one peptide, AKH that created much more regular clusters, differentiating between sugar and liver-fed flies (Fig. 3.4).

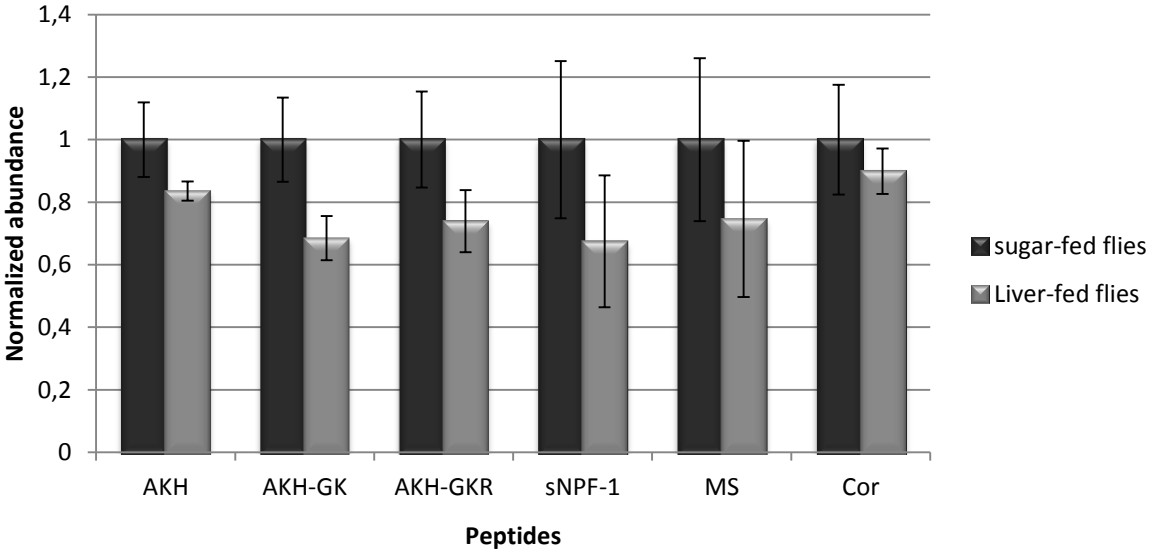


Fig. 3.4 Differential distribution of six peptides present in the CC of sugar-fed and liver-fed flies, (measured after 5 hours ppf). The data is normalized against the amount of the particular peptide measured in sugar-fed flies. The amount of each peptides decreases after liver feeding. Abbreviations: AKH, adipokinetic hormone; Cor, corazonin; MS, myosupressin; sNPF, short neuropeptide F (four replicates of 50 pooled CCs each); mean *± SD.*

More detailed analysis presenting the specific numeric values of the particular peptides is presented in the table 3.2.

Peptide name	$[M+H]^+$	Mass error [ppm]	Average amount of condition 1	Average amount of condition 2	Sequence	Ratio cond. 2/1	P val
AKH	975.45	2.34	8928380	7455855	pQLTFSPDWa	0.84	0.21
AKH-GK	1161.55	0.65	13368794	9154103	pQLTFSPDWGK	0.68	0.50
AKH-GKR	1317.66	0.39	2692335	1990605	pQLTFSPDWGKR	0.74	0.17
sNPF-1	974.59	1.02	2817385	1900240	SPSLRLRFa	0.67	0.13
MS	1247.66	0.72	985075.7	735358.6	TDVDHVFLRFa	0.75	0.23
Cor	1369.64	6.38	7396.215	4368.73	pQTFQYSRGWTNa	0.59	0.91

Table 3.2 Detailed analysis presenting numeric values of each peptide in both tested conditions. Condition 1 refers to CC peptidergic extract of sugar-fed flies, whilst condition 2 refers to CC peptides content extracted from flies 5 hours ppf. Table contains calculated ratio value between condition 2 and 1. The value <1 indicates decrease/release of the particular in liver-fed flies. Abbreviations: AKH, adipokinetic hormone; Cor, corazonin; MS, myosupressin; sNPF, short neuropeptide F (four replicates of 50 pooled CCs each); mean *± SD.*

All peptides were present in the peptide extract in several forms of different intermediates, different isotopes and different posttranslational modifications (Supp. fig. 3.2). AKH was detected in 16 different forms (Supp. fig. 3.1), which resulted in ten sequences (pQLTFSPDWGKR; pQLTFSPDWGK; pQLTFSPDWa; pQLTFSPDWGKRSGNTFDQPG; QLTFSPDWGK; LTFSPDWGK; TFSPDWGK; TFSPDWa; FSPDWGK; FSPDWa) representing the peptide at different processing/maturation stages (Fig. 3.5).

Fig. 3.5 All ten detected peptide sequences of the *S. crassipalpis* AKH situated in the *D. melanogaster* AKH precursor (UniProt Acc. No. **GMOY003470**). Sequence marked in grey is identified as a partial *S. crassipalpis* AKH precursor identical to the *D. melanogaster* sequence. Bleu 'p' and orange 'a' stand for pyroglutamine and amidation.

3.3.3 In vivo *stimulus of adipokinetic hormone on midgut activity*

To further validate our mass spectrometry based prediction that AKH is an elicitor/enhancer of proteolytic activity in the midgut of liver-fed anautogenous flesh flies, we tested different AKH doses, in order to determine the most adequate dose that restores normal physiological digestion process in liver primed decapitated females.

For that purpose, flies were first fed with liver, decapitated after 30 min and subsequently injected with different amounts of AKH (0 ng, 10 ng, 100 ng, 500 ng, 1000 ng corresponding to 0 mM, 0.01 mM, 0.1 mM, 0.51 mM, 1,03 mM) dissolved in Ringer's solution containing 10% DMSO (necessary for proper dissolving of AKH). The enzymatic activity was measured at 4 hours ppf (the moment of the highest proteolytic activity observed in the intact liver-fed flies) (Fig.3.6). The obtained results were compared to the positive control of non-decapitated liver-fed flies. It is clear that starting from a dose of 100ng of AKH (0.1mM), the induced total proteolytic activity approaches were similar to the activity level measured in the liver-fed positive control flies (Fig. 3.7). For that reason, in our further experiments we kept using 100ng (0.1 mM) AKH dose for injection.

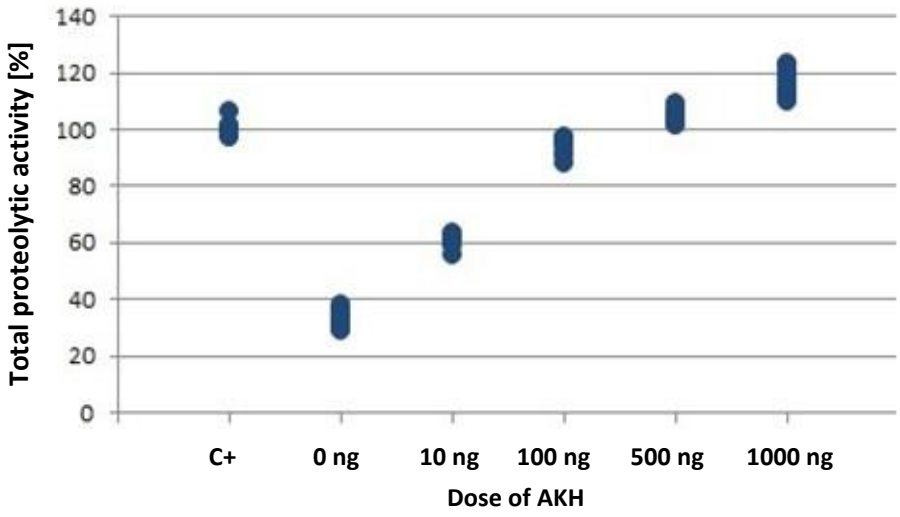


Fig. 3.6. Dose response effect of different doses of AKH injected into decapitated liver-fed flies, measured at 4 hours ppf. The result of the proteolytic activity that is the most similar to the positive control (C+) of the intact liver-fed flies is caused by the injection of 100 ng (0.1 mM) AKH (*n= 10 individual organisms*).

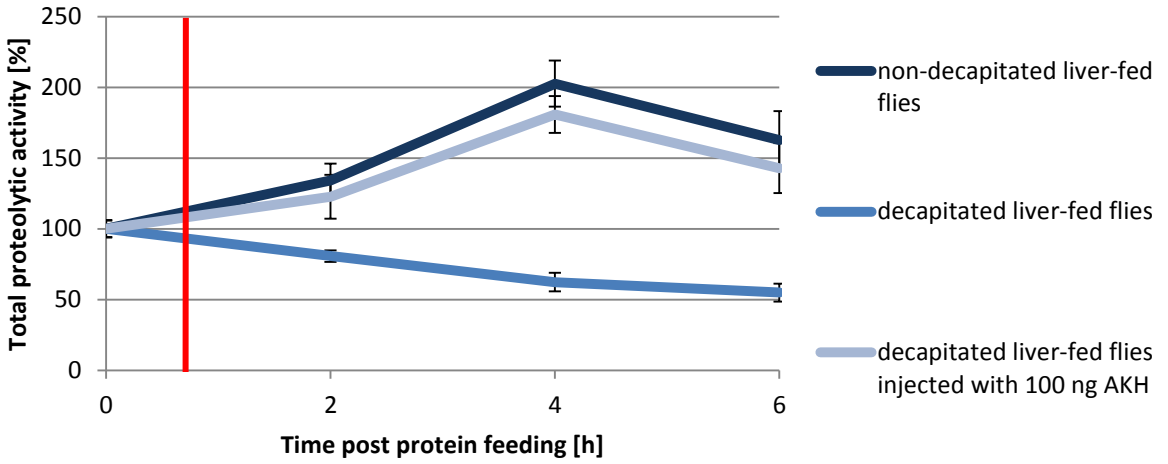


Fig. 3.7 Midgut proteolytic activity profile of decapitated liver-fed flies injected with 100 ng (0.1 mM) AKH compared to the proteolytic activity profile of both positive control of liver-fed flies and negative control of decapitated liver-fed flies. Red line indicates the end of feeding, decapitation and injection moment (four biological replicates of three pooled midguts each); mean *± SD.*

One hundred nanograms of AKH (0.1 mM) was also injected into non-decapitated sugar-fed flies. The digestive activity of those flies also increased and that time, the measured proteolytic activity increased constantly, showing no dip after a 4 hours ppf peak. That was most probably due to the lack of competing liver proteins in the samples (Fig. 3.8).

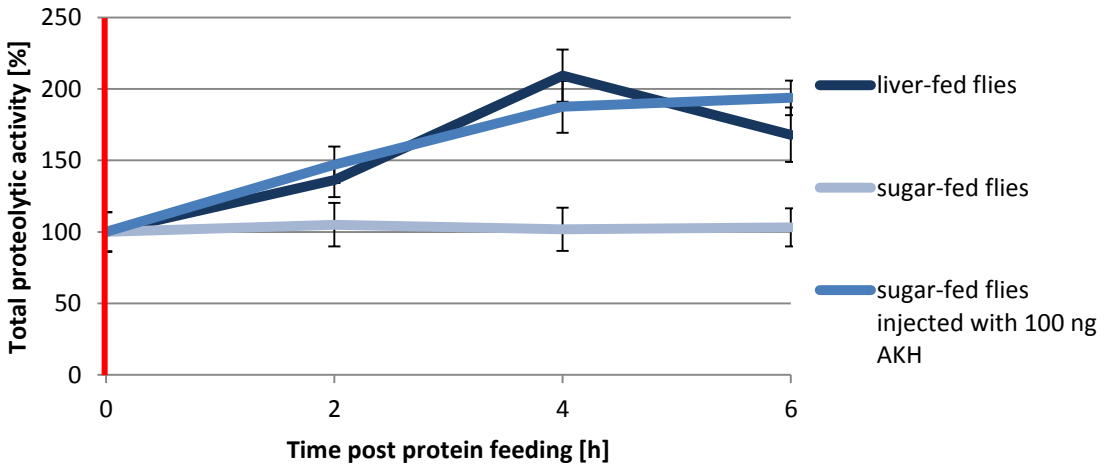


Fig. 3.8 Midgut proteolytic activity of non-decapitated sugar-fed flies injected with 100 ng (0.1 mM) AKH. The peptide is also able to stimulate digestion in sugar-fed insects; no liver priming is needed. Red line indicates injection moment (four biological replicates of three pooled midguts each); mean *± SD.*

To determine if AKH stimulates midgut digestive activity in a direct way, three midguts (dissected from liver-primed flies) were incubated in 60 μl of Ringer's solution, containing 1 μM – 40 μM of AKH. After 2 hours of incubation time, samples were homogenized and the proteolytic digestive activity was measured, as described in paragraph 3.2.10.

Unfortunately, the direct incubation of the midguts (Fig. 3.9) in the medium supplemented with AKH did not show any stimulating activity within the tested concentration series. The level of the midgut proteolytic activity observed in that experiment was almost the same in all samples and statistical analysis confirmed the lack of any significant difference between the negative control containing 0 μM AKH and the other samples.

The extended sample content, up to the whole digestive tract, gave the same negative results (data not shown).

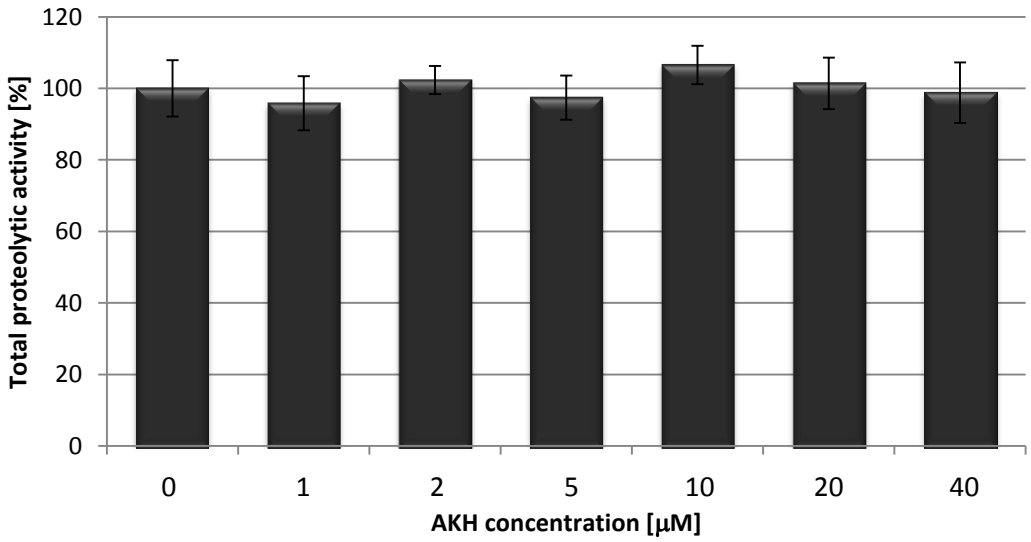


Fig. 3.9 Proteolytic activity of the midgut samples directly incubated in different concentrations of AKH. No stimulatory AKH activity is observed in any tested concentration (four biological replicates of three pooled midguts each); mean *± SD.*

Based on the literature, the midgut-AKH incubation samples were supplemented with a variety of additional tissues that were recognized as having the AKH receptor expressed. Both organs (midgut and additional tissue) were always taken from the same liver-primed fly.

The enrichment of the incubating mixture by the addition of fat body, which is well known to be able to respond to the AKH stimulus, did not result in any consequent increase of the proteolytic activity (Fig. 3.10). Furthermore, the supplementation with that particular tissue leaded to a large unexplainable variation between different conditions. Samples incubated in the medium containing 5 µM and 20 µM AKH showed much higher enzymatic activity than samples that were incubated in the medium containing 1 µM, 2 µM, 10 µM and 40 µM of AKH. All three independent experimental replicates always resulted in different variations between different conditions, making the results unreproducible.

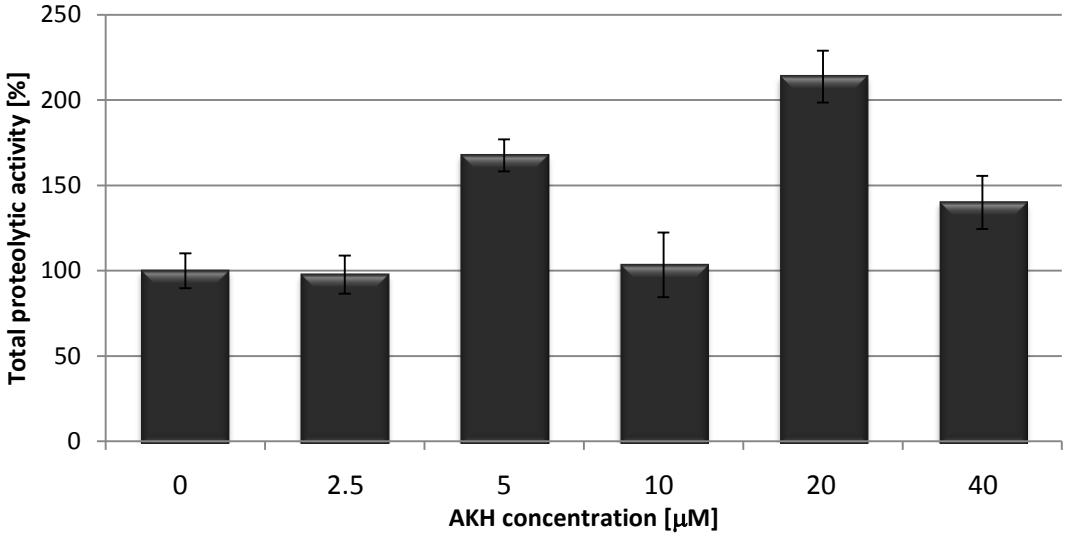


Fig. 3.10 Proteolytic activity of the midgut samples supplemented with the additional tissue of the fat body incubated in different concentrations of AKH. No stimulating AKH activity is observed in any tested sample (four biological replicates of three pooled midguts each); mean *± SD.*

In the second series of experiments, the midgut incubation mixture was supplemented with ovaries as putative AKH intermediate responder, but again no increase in digestive activity was observed (Fig. 3.11). The same negative results were observed in the last gut-AKH incubation sample which was enriched with fly brains (data not shown).

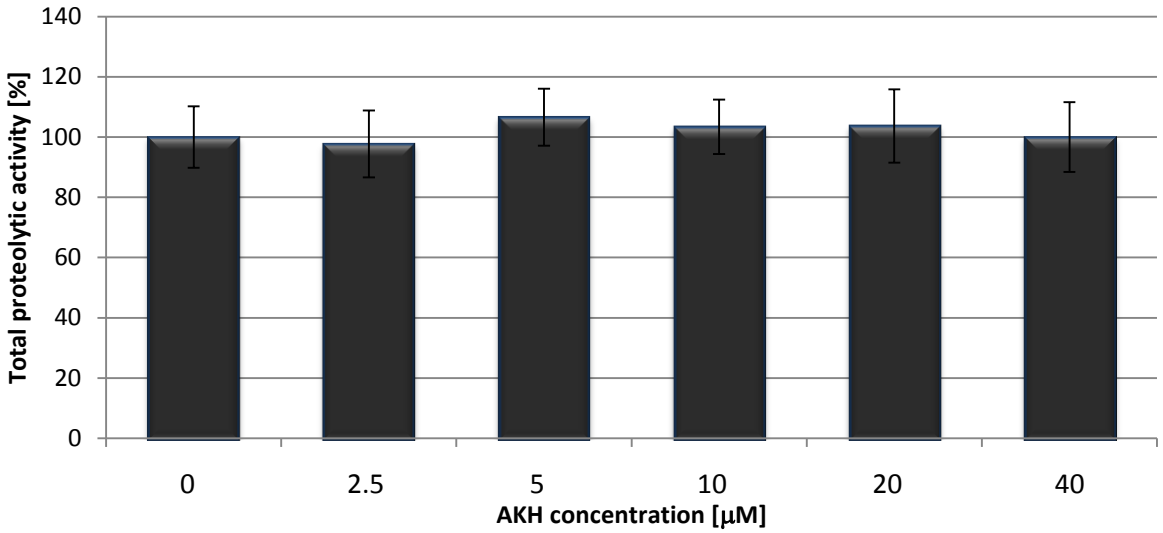


Fig. 3.11 Proteolytic activity of the midgut samples supplemented with the additional tissue of ovaries incubated in different concentrations of AKH. No stimulating AKH activity is observed in any tested sample (four biological replicates of three pooled midguts each); mean *± SD.*

3.3.4 Adipokinetic hormone immunostaining

A specific antibody staining was clearly proving the presence of the AKH peptide in CC that was situated between esophagus and proventriculus (Fig.3.12). AKH, as expected, seemed to be aggregated in the big storage parts of this neurohemal organ.

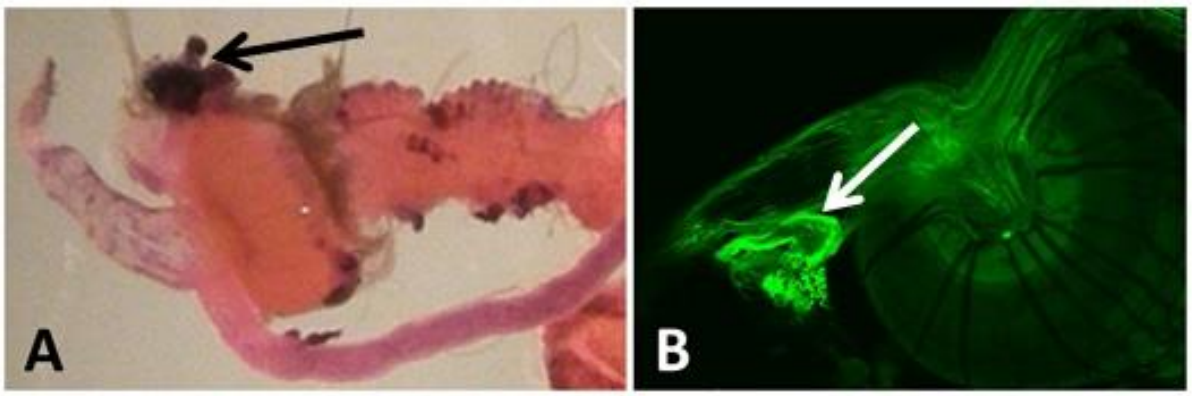


Fig. 3.12 Localization of adipokinetic hormone in of whole mount tissue of sugar fed flesh flies. Peroxidase-Shu coloring immunostaining of the adipokinetic hormone present in the *corpora cardiaca* attached to proventriculus (A) and fluorescent immunocytochemistry of the same neuropeptide in the retrocerebral complex affiliated to the esophagus (B). Arrows are indicating the positive signals.

3.3.5 The effect of the additional CC derived peptides on digestion of sugar-fed flies

The peptidomics analysis revealed that CC also stored some other neuropeptides known to be involved in digestion control: two forms of short neuropeptide F, corazonin and myosypressin. To make sure that their unability to create a regular cluster in the quantitative, differential analysis was not accidental and that they did not play any role in enzymatic regulation in *S. crassipalpis*, all four molecules were tested in both sugar and liver-primed flies.

Injections of 1 μg (0.8 mM) short neuropeptide F-1 (Fig. 3.13 sNPF-1), 1 μg (1.1 mM) short neuropeptide F-3 (Fig. 3.13 sNPF-3) and 1 μg (0.7 mM) corazonin (Fig. 3.13 Cor) did not show any effect upon the enzymatic activity in sugar-fed flies. Nevertheless, the same injected dose of myosuppressin (1 μg corresponding to 0.8 mM) stimulated the early phase of proteolytic activity (Fig. 3.13 MS). An increase in measured enzymatic activity level was observed at 2 hours ppf that afterwards dropped again to the basal 'resting' level. The result was reproducible. Nevertheless, any other injected doses of 100 ng (0.08 mM) and 5 μg (4 mM) myosuppressin were unable to stimulate digestion for longer period of time or higher activity level (data not shown).

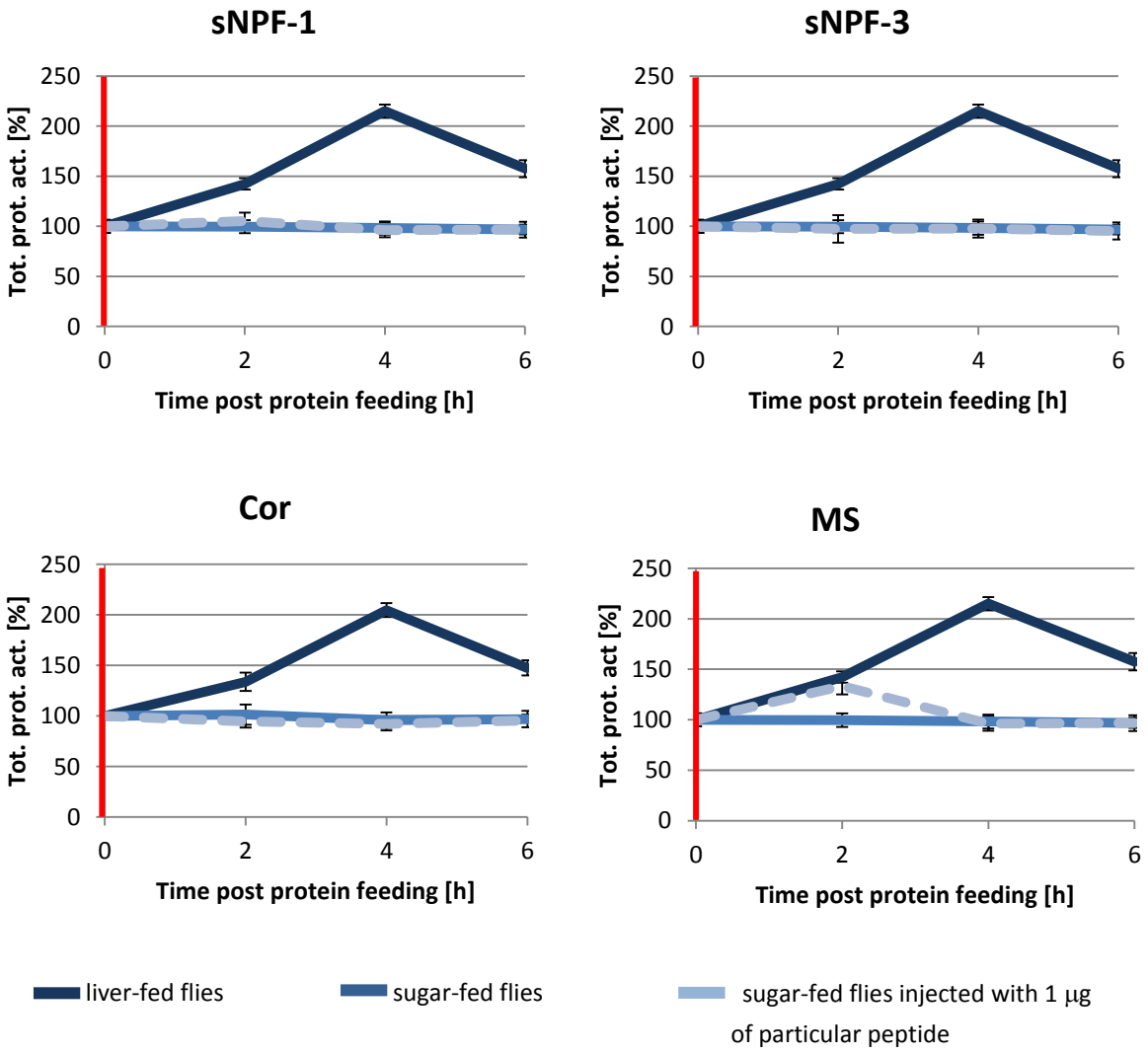


Fig. 3.13 The influence of 1 µg (0.8 mM) short neuropeptide f-1 (sNPF-1), 1 µg (1.1 mM) short neuropeptide F-3 (sNPF-3), 1 µg (0.7 mM) corazonin (Cor) and 1 µg (0.8 mM) myosuppressin (MS) on midgut proteolytic activity in sugar-fed flies. Only myosuppressin injections show a reproducible stimulatory effect observed at 2 hours ppf. Red lines indicate the injection moment (four biological replicates of three pooled midguts each); mean *± SD.*

3.3.6 The effect of the additional CC derived peptides on digestion of decapitated liver - primed flies

The same analysis (digestion stimulation potential of all other peptides found in CC) was performed on decapitated liver-fed flies. Injection of short neuropeptide F-1 (Fig. 3.14 sNPF-1) and corazonin (Fig. 3.14 Cor) had no stimulating effect on digestive enzymes in those insects. In both cases, measured proteolytic activities even overlapped the results presented

by the negative controls of decapitated liver-fed flies. A different situation was observed in the decapitated liver-fed females injected with either short neuropeptide F-3 (Fig. 3.14 sNPF-3) or myosuppressin (Fig. 3.14 MS). Early after injection, the digestive activity in both treated groups was slightly elevated but this stimulating effect was not permanent as the enzymatic activity decreased to a level of 20-30% at 10 hours ppf (data not shown).

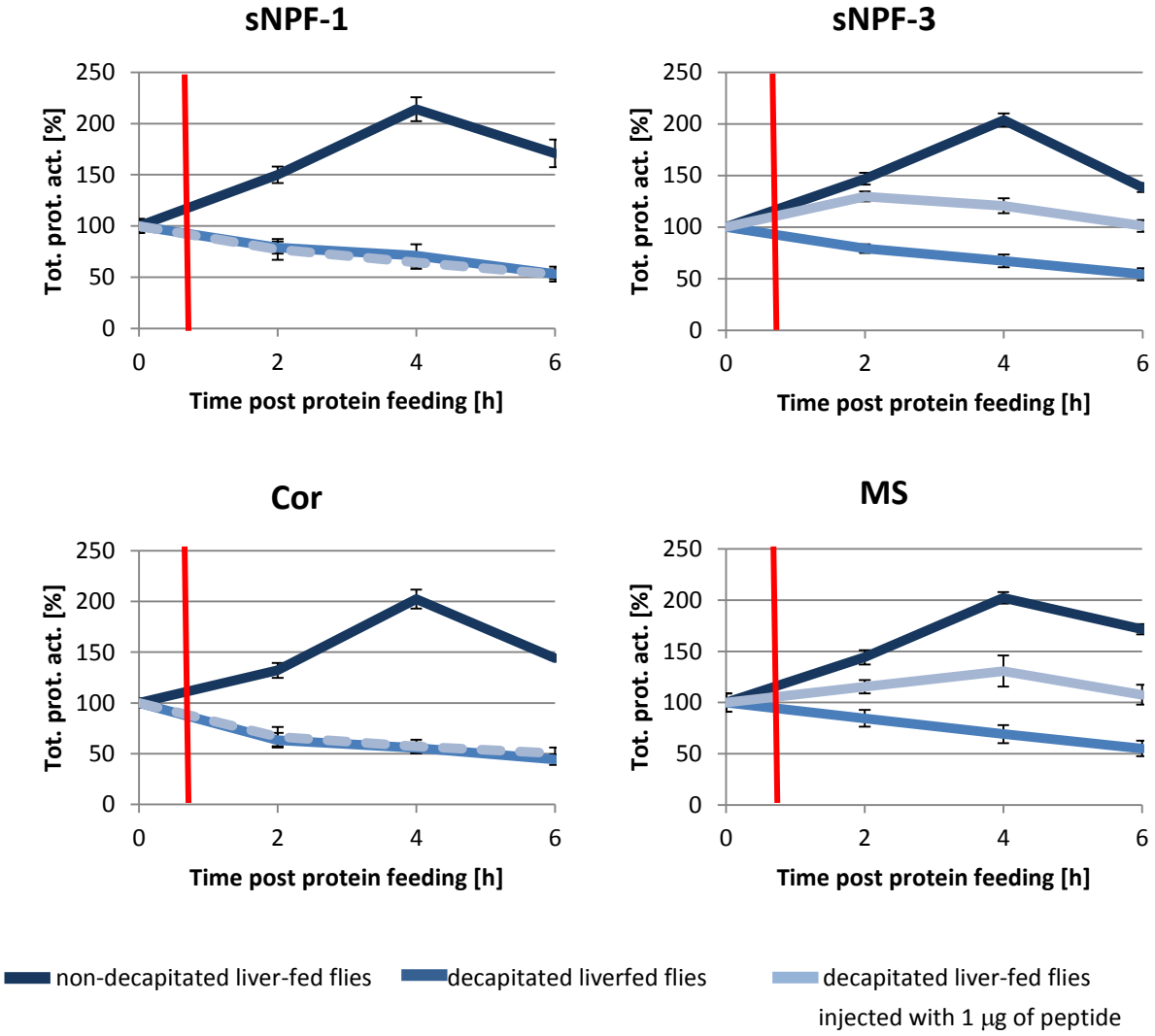


Fig. 3.14 The influence of 1 µg (0.8 mM) short neuropeptide f-1 (sNPF-1), 1 µg (1.1 mM) short neuropeptide F-3 (sNPF-3), 1 µg (0.7 mM) corazonin (Cor) and 1 µg (0.8 mM) myosuppressin (MS) on midgut proteolytic activity of decapitated liver-fed flies. Short neuropeptide F-3 and myosupressin show some slight midgut stimulatory activity. Red lines indicate the end of feeding, decapitation and injection moment (four biological replicates of three pooled midguts each); mean *± SD.*

Simultaneous injections of 100 ng (0.1 mM) AKH and 1 µg (1.1 mM) short neuropeptide F-3 (Fig. 3.15) or myosuppressin (data not shown) did not show any synergistic effects of the two mixed compounds. The enzymatic activity profile of flies injected with two neuropeptides looked the same as the one observed for the flies that were only injected with AKH (p_2=0999986.; p_4=0.995728; p_6=0.992863).

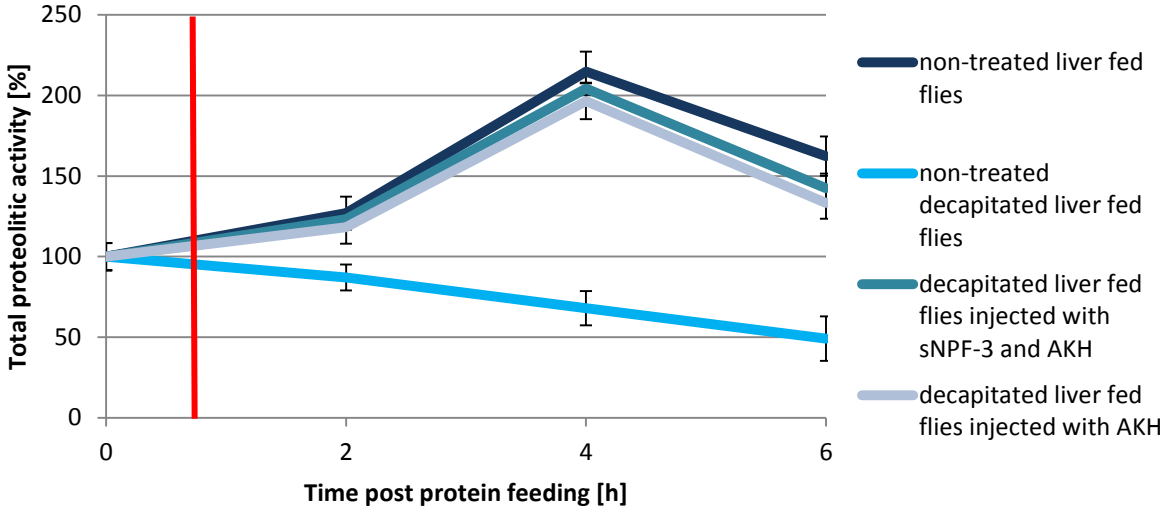


Fig. 3.15 The influence of the AKH treatment supplemented with sNPF-3 compared to single AKH injection into decapitated liver-fed flies. There is no synergistic effect of two injected compounds compared to a single AKH injection. Red line indicates the end of feeding time, decapitation and injection moment (four biological replicates of three pooled midguts each); mean *± SD.*

3.4 Discussion

The CC represents a major neurohaemal organ involved in neurosecretion. Apart from its storage compartment, it also contains a glandular part that is responsible for the intrinsic peptides synthesis. Neuropeptides present in the CC of *Sarcophaga crassipalpis* are: two forms of short neuropeptides F, myosuppressin, corazonin, pyrokinin, CAPA and AKH. Almost all of these molecules are in some way related to feeding/digestion behaviors, which means that they all can be involved in the stimulation of the digestion process.

Unfortunately, it was not possible to identify all detected signals in the CC mass spectrometry spectrum. The unknown peak of 1457.89 m/z was previously detected by

other researchers, as unidentifiable signal in the related flesh fly, *Neobellieria bullata* (Rahman *et al*., 2013). This peak also appeared in the MALDI-TOF spectrum of the blowfly, *Protophormia terraenovae*, reared under similar conditions. However it disappeared in the mass spectrometry analysis of these flies that were reared under diapause-including conditions (*Inosaki et al., 2010*). It is suggested that this unidentified peak is rather not involved in any digestion/feeding behavior, which means that this molecule does not have to be taken into account in our analysis.

Our differential peptidomics results could not reveal the experimentally predicted 100% differential changes (total release) of any detected peptide, as all peptides were always detected in both sugar and liver-fed insects. Nevertheless, the replicates of the AKH molecule form two different clusters for both studied conditions. The difference in AKH amounts between CC from sugar and liver-fed flies is large and indicates that about 27% of the fully mature peptide is released after the protein meal, whilst the normal release level of this peptide in non-related locusts ranges between 2-6% (Diederen *et al.*, 2002). Although AKH does not stimulate proteolytic activity of isolated midguts *in vitro* (nor in combination with other organs containing an AKH receptor), the evidenced *in vivo* dose-response displays an optimum activity dose of 100 ng (0.1 mM) AKH per fly. This substantiates our findings, as do the findings of AKH that affect enzymatic activity in the gut of the cockroach, *Periplaneta americana* (Bodláková *et al.*, 2016), and in both the midgut and salivary glands in the firebug, *Pyrrhocoris apterus* (Kodrik *et al.,* 2012;Vinokurov *et al.,* 2014). Additionally it has been also recently found that AKH stimulates crop contractions and consequently food motility into the midgut in the blowfly *Phormia regina* (Stoffolano *et al.*, 2014), which has a direct impact on digestion, as well as on sugar and amino acid raises in the hemolymph.

The immunocytochemistry staining confirms the large scale abundance of the AKH peptide in the neurohemal organ, CC, as is well described in the literature for many other insects (Gäde & Auerswald, 2003;Abdel-Latief & Hoffmann, 2007;Diederen *et al.*, 2002;Rahman *et al.*, 2013).

AKH is structurally related to the mammal gonadotropin-releasing hormone (GnRH) (Hauser *et al.*, 1998) that is involved in the control of vertebrate reproduction. It regulates the synthesis and the release of the gonadotropins luteinizing hormone and follicle-

stimulating hormone that stimulates the biosynthesis of several gonadal steroids (Kah *et al.*, 2007). A protostomian GnRH homologue has first been identified in the molluscan species of *Octopus vulgaris* and is reminiscent of both insect AKH and vertebrate GnRH (AKH-GnRH) in the nematode *Caenorhabditis elegans* (Iwakoshi *et al.*, 2002;Lindemans *et al.*, 2009). In contrast to the molluscan GnRH-like peptide that is only involved in reproduction, the nematode AKH-GnRH seems to be involved in both, reproduction and energy metabolism (Ashrafi *et al.*, 2003;Lindemans *et al.*, 2009).

As already mentioned, AKH is mainly known in insects for its energy mobilization function. Nevertheless, Abdel-Latief et al. (2004) suggested that one of the AKH peptides would regulate oocyte maturation in the fall armyworn, *Spodoptera frugiperda*. Importantly, this peptide is characterized by a unique amino acid sequence, which is different from the other AKHs found in this insect. Other researchers demonstrated that AKH can have an indirect effect on egg formation, which could be the consequence of an induced interruption of the energy storage (in the fat body) of the cricket, *Gryllus bimaculatus* (Lorenz, 2003).

The peptidomics data of this study focuses only on CC-derived peptides within the range below 1.5 kDa and the further exploration of the higher molecular mass ranges of peptides/small proteins, present in the acidic methanol/acidic water CC extract, failed (probably due to the lack of a correct purification protocol). A successful method of sample preparation might allow the additional discovery of larger molecules, having among them the predicted, but never traced flesh fly specific gonadotrophin that might have similarities to the ovary ecdysteroidogenic hormone (OEH), which so far has only been identified in mosquitoes(Brown & Cao, 2001).

To avoid any confusion and doubt that some other peptide, that is known for its feeding/digestion control, is also involved in the stimulation of the enzymatic activity in *Sarcophaga crassipalpis*, all of these peptides were additionally tested for their putative stimulating effect on the measured digestive activity. In sugar-fed flies, only myosuppressin caused some stimulatory effect, which unfortunately was of a short-term nature. In liver-fed flies, short neuropeptide F-3 also showed some positive stimulus. This is in contrast to the findings reported in the cockroach, *Periplaneta americana,* in which species short neuropeptide F inhibits protease activity (Mikani *et al.*, 2012). Even if these two peptides

(myosuppressin and short neuropeptide F-3) show some small enzymatic stimulatory influence, they definitely do not synergistically enhance the AKH-elicited effect.

Not only peptides but also protein hormones can be involved in the regulation of the digestive process. Late trypsin activity can be directly up-regulated by OEH and by insulin-like peptide 3 (ILP-3) in blood fed mosquito, *Aedes aegypti,* and this insulin activity is synergized by the presence of free amino acids (Gulia-Nuss *et al.*, 2011). Following the completion of vitellogenesis, trypsin activity can be post-transcriptionally down regulated by AeaTMOF in mosquito and by NebTMOF in the flesh fly, *Neobellieria bullata* (Borovsky *et al.*, 1996).

CHAPTER 4.

Molecular cloning and characterization of the adipokinetic hormone receptor of the anautogenous flesh fly, *Sarcophaga crassipalpis*‡

‡ Parts of this chapter are published in: Bil,M.; Timmermans,I.; Verlinden,H.; Huybrechts,R. (2016) Characterization of the adipokinetic hormone receptor of the anautogenous flesh fly, *Sarcophaga crassipalpis*. Journal of Insect Physiology (accepted)

4.1 Introduction

Adipokinetic hormone (AKH) is one of the best studied neuropeptides in insects. Its physiological function is mediated by the adipokinetic hormone receptor (AKHR) which represents a seven transmembrane domains G protein-coupled receptor (GPCR). AKHR was first identified and pharmacologically characterized simultaneously in the fruit fly, *Drosophila melanogaster*, and in the silkworm, *Bombyx mori* (Staubli *et al.*, 2002). Upon ligand binding, AKHR has been demonstrated to couple through G_q protein, resulting in an increase of intracellular Ca^{2+} in the cockroach *P. americana*, mosquito *A. gambiae* and flies *D. melanogaster* and *G. morsitans* (Hansen *et al.*, 2006;Belmont *et al.*, 2006;Caers *et al.*, 2015;Caers *et al.*, 2012), and through G_s, causing increased intracellular cAMP levels in the silkworm, *Bombyx mori* (Zhu *et al.*, 2009;Shi *et al.*, 2011). Due to the functional specificity of AKH, in regard to the process of energy household, AKHR is particularly abundant in the fat body of the insects.

Some studies determining the structural specificity of the ligand-receptor binding indicates several amino acid residues of the AKH peptide ligand that are crucial for the AKHR activation: an aromatic amino acid at the fourth position, which contains a phenyl ring (phenylalanine or tyrosine) and tryptophan at the eighth position, which contains an indole ring and the entire N-terminal pentapeptide. Neither the amino acid transitions at the sixth

nor the seventh positions of this octapeptide, have any significant impact on receptor activation (Gäde, 1990;Gäde & Hayes, 1995;Velentza *et al.*, 2000).

Flies are described as insects of carbohydrate-base metabolism, where AKH stimulates the release of carbohydrates (trehalose) into the hemolymph (Gäde *et al.*, 1990). Some first molecular studies concerning this mode of action prove that AKH, as a ligand binding its receptor, changes its conformation and interacts with GTP-binding proteins (G-proteins) (Fig. 4.1). The signal is further transduced via G_s protein to adenylyl cyclase, which amplifies the message by the production of intracellular cyclic adenosine monophosphate (cAMP) as the secondary messengers. cAMP binds to the AMPK protein kinase which further activates glycogen phosphorylase. Activated glycogen phosphorylase ultimately converts glycogen into metabolized trehalose (reviewed by Gäde & Auerswald, 2003). This pathway explains why fly AKH got its synonym name of hypertrehalosaemic hormone (HrTH) (Gäde et al., 1990).

Furthermore, the AKHR binding mediated action, by means of G_q protein, also activates phospholipase C and results in inositol triphosphate (IP_3) and diacylglycerol (DAG) production (Auerswald & Gäde, 2002). IP_3 binds to specific receptors of the endoplasmic reticulum (ER), and induces the release of calcium ions from the ER lumen into the cytosol (Vroemen *et al.*, 1995) which in some way, also stimulates glycogen phosphorylase and trehalose synthesis(reviewed by Gäde & Auerswald, 2003).

The last AKHR mediated pathway uses DAG produced by phospholipase C that, together with the increasing amount of the calcium ions, activates different forms of protein kinase C. One of these forms directly phosphorylates phosphorylase *b* kinase, which also stimulates glycogen phosphorylase. The other form of protein kinase C activates phospholipase A_2 that converts phospholipids into free unsaturated fatty acids, again necessary for trehalose production. This latter pathway has so far only been observed in the cockroach, *Periplaneta americana* (Sun & Steele, 2002a;Sun & Steele, 2002c;Sun & Steele, 2002b).

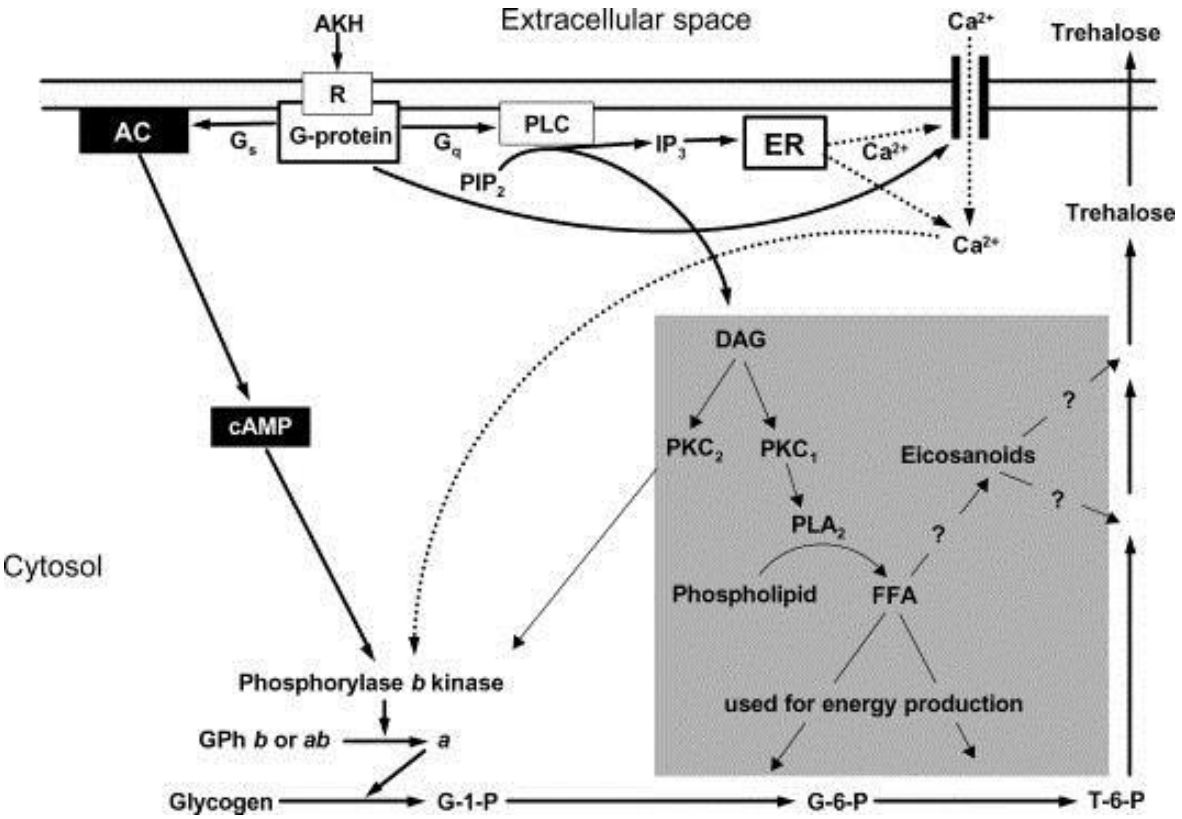


Fig. 4.1 Schematic signal transduction mediated by AKH (hypertrehalosaemic mode of action) and AKHR in the insect's fat body. AKHR activation results in synthesis and release of trehalose that can be mediated through cAMP/calcium/DAG mediated pathway. Abbreviations: AC, adenylyl cyclase; AKH, adipokinetic hormone; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; ER endoplasmic reticulum; FFA, free fatty acids; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; GPh, glycogen phosphorylase; IP_3, inositol triphosphate; PIP_2, phosphatidylinositol biphosphate; PLA_2, phospholipase A_2; PKC, protein kinase C; PLC, phospholipase C; R, receptor; T-6-P, trehalose-6-phosphate. Signaling pathway located in the grey area is specific for *P. americana*. Image credits: (Gäde & Auerswald, 2003)

In this chapter the unknown DNA sequence of *Sarcophaga crassipalpis* AKHR was identified, analyzed and cloned. This was done in order to better understand the possible mechanism of the AKH action and its digestion stimulating activity. Furthermore, that receptor was expressed in the Chinese hamster ovary (CHO-WTA11) cell lines that stably express a promiscuous $G_{\alpha 16}$ protein for pharmacological characterization of different ligands which interact with this receptor. Ligand-receptor interaction resulted in activation of $G_{\alpha 16}$ which always gives an increase in intracellular calcium ion levels and does not provide information about the *in vivo* used downstream signaling pathway(s). On the other hand, the data which

relates to tissue-specific AKHR expression under different nutritional regimes in anautogenous adult female flesh flies, provides some essential information about the *in vivo* target sites of AKH. The reported changes in target site receptor expression levels in accordance with the different feeding conditions can be directly related to the fly's reproductive capacity as only protein-fed anautogenous flesh flies develop eggs and offspring.

4.2 Materials and methods

4.2.1 Insect rearing

Flies used for experiments were reared as described in chapter 2, paragraph 2.2.1.

4.2.2 Feeding procedure

Strict feeding procedure was the same as described in chapter 2, paragraph 2.2.4.

4.2.3 RNA isolation and cDNA synthesis

Sixty sugar-fed female flies were used for the experiment and subsequently divided ad random into two groups. The first group of insects remained under sugar and water diet, whilst the second group was offered a piece of cow liver for 45 minutes. Five hours after the beginning of the feeding time, seven different tissues including brain, foregut, midgut, hindgut, fat body, ovaries and Malphighian tubules were dissected with great care in *Sarcophaga* Ringer's solution. For each condition, three biological replicates of ten different sugar or liver-fed fly tissues were collected. RNA isolation and cDNA synthesis procedures were executed as described in chapter 2, paragraph 2.2.6.

4.2.4 Identification of the partial adipokinetic hormone receptor sequence by Polymerase Chain Reaction (PCR)

Because the genome of *Sarcophaga crassipalpis* is unknown and at the beginning it was not possibility to find any fragment of a putative SarcrAKHR in the transcriptome, there was no possibility to design full length gene specific PCR primers. Different PCR primer pairs (Table 4.1) were designed based on the best conserved regions of flies' AKHR sequences. Three

related species with a complete genome available, namely *Drosophila melanogaster* (GenBank Acc. No. **NM_205917.3**), *Glossina morsitans* (GenBank Acc. No. **HQ640948.1**) and *Musca domestica* (GenBank Acc. No. **NM_001309065.1**) were aligned using the online tool Clustal Omega provided by EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/) and analyzed for the best regions for primers design (Suppl. Fig. 1). Each PCR reaction mixture contained 0.5 μl Advantage II polymerase mix (Clontech), 5 μl 10x Advantage PCR buffer (Clonetech), 1 μl nucleotides mixture (10 mM each), 1 μl forward and reverse primers (10 μM), 38.5 μl MQ and 3 μl cDNA of the fat body. The amplification PCR program contained an initial denaturation step of 95^{o}C for 180 s following by 30 cycles of 94^{o}C for 30 s, gradient of 50^{o}C -70^{o}C for 60 s, 68^{o}C for 60 s and a final elongation step of 68^{o}C for 600 s. The amplification of the product took place in three lowest annealing temperatures of 50.0^{o}C, 54.0^{o}C and 58.8^{o}C for the combination of a forward primer of *D. melanogaster* AKHR 3 (5'- GCCATCGCCGATCTAAT-3') and a reverse primer of *M. domestica* AKHR 3 (5'-ATCATCGTTGGAACGGC-3').

All primers described in this chapter for all PCR reactions were prepared by Sigma-Aldrich Company.

Name	Forward primer sequence	Reverse primer sequence
Drome1	5'-TGTATCTGCTGACCAAGC-3'	5'-ACAATCACAATCGTGATGGT-3'
Drome2	5'-GTATTGACATCATGCTAATGCA-3'	5'-GTCCAGCAGATGATGAA-3'
Drome3	5'-GCCATCGCCGATCTAAT-3'	5'-GTCATCGTTGGAACGC-3'
Glomo1	5'-TTGCTTACTAAGCGCGT-3'	5'-AGACAATGACAATAGTGATAGTCAT-3'
Glomo2	5'-GCGTATTGATATTATGTTAATGCAT-3'	5'-TGTAATAAGGCGTCCAGC-3'
Glomo3	5'-CGCGTATTGATATTATATGTTAATG-3'	5'-ACATCATCGTTTGAACGAC-3'
Musdo1	5'-TGTATCTGCTGACCAAGC-3'	5'- ACAATCACAATGGTTATGGTCAT-3'
Musdo2	5'-GTATTGATATCATGCTAATGCAT-3'	5'-GTCCAGCAGATGAGGAA-3'
Musdo3	5'-TGCATTTGGCAATAGCCG-3'	5'-ATCATCGTTGGAACGGC-3'

Table 4.1 All primer pairs used to pick up the AKHR sequence. The matching primers are Dromo3 forward and Musdo3 reverse. Primers designed on conserved regions of *Drosophila melanogaster*, *Glossina morsitans* and *Musca domestica*.

Furthermore the results of the PCR reactions were verified using 1% agarose gel electrophoresis (100V/100mA for 1h). All obtained products of the correct expected length were purified using GenElute Gel Extraction Kit (Sigma-Aldrich) and cloned into a pCR4-TOPO TA sequencing vector (Invitrogen). The cloned vectors were transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen). The cells were grown overnight at 37 ^{o}C on Luria-Bertani (LB) agar plates (35 g/l, Sigma-Aldrich) with ampicillin (50 μg/ml, Invitrogen). Single colonies were transferred into LB liquid medium (25 g/l, Sigma-Aldrich) with ampicillin (100 μg/ml) and grown overnight at 37^{o}C in the shaking incubator. The plasmids were isolated using the GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich) and sequenced using M13 primers unless stated otherwise (LGC Genomics, Germany).

4.2.5 Full length cloning of the adipokinetic hormone receptor sequence

To establish the N-terminal encoding cDNA sequence, a forward primer was designed based on the short transcriptome sequence of *Sarcophaga crassipalpis* (Hahn transcriptome: **HAHN.FLY.6451.C1**) and genomic sequence data of *Neobelliera bullata,* former called *Sarcophaga bullata* (GenBank Acc. No. **JXPI0114051.1**).

Rapid Amplification of cDNA Ends (RACE) PCR was performed in order to determine the cDNA encoding the C-terminal end of the receptor.

The oligodT anchor primer (5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT-3') necessary for RACE PCR was already added at the level of cDNA synthesis (instead of random hexamer primers). The PCR mixture contained 0.5 μl Advantage II polymerase mix (Clontech), 5 μl 10x Advantage PCR buffer (Clonetech), 1 μl nucleotides mixture (10 mM each), 1 μl gene specific (5'-TCATTTGCAGCGAATTTCACA-3') (12.5 μM) and anchor primers (5'-GACCACGCGTATCGATGTCGAC-3') (37.5 μM), 38.5 μl MQ and 3 μl undiluted fat body cDNA. The used amplification program had an initial denaturation step at 95^{o}C for 180 s following 30 cycles at 94^{o}C for 45 s, 60^{o}C for 60 s, 68^{o}C for 60 s and final elongation step of 68^{o}C for 600 s.

The PCR product was size controlled, cloned and sequenced as described above.

4.2.6 Quantitative tissue distribution analysis of the adipokinetic hormone receptor

The AKHR gene transcript level was measured using the quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) using primers (AKHR forward: 5'-CGTTGCCTGGTGTAGTTCCT-3' and AKHR reverse: 5'-GGATGCTCCTCCAAGTGGAA-3'). The rest of the procedure was as described in chapter 2, paragraph 2.8.

Qbase+ software v.2.4 analysis determined three housekeeping genes to be the most stably expressed and those genes were used as reference genes (elongation factor 1α, heat shock protein 90 and glyceraldehyde 3-phosphate dehydrogenase) (Supp. fig. 4.2).

4.2.7 Cloning of the adipokinetic hormone receptor into the TOPO TA expression vector

The full AKHR sequence (GenBank Acc. No. **KU640386)** was amplified by PCR reaction using specific forward (5'-CACCATGACAGAGTCCGAGATAA-3') with the 'CACC' Kozak sequence added to the 5' side of the primer and reverse (5'-TTATGTTTTTTGTTGTAGATTAG-3') primer. The PCR mixture was composed as described in 2.3 paragraph. The amplification program consisted of an initial denaturation step of 95^{o}C for 180 s, following 35 cycles at 94^{o}C for 30 s, 60^{o}C for 60 s, 68^{o}C for 120 s and a final elongation step at 68^{o}C for 600 s. The analyzed and purified PCR product was subsequently cloned into a pcDNA3.1/V5-His-TOPO TA expression vector (Invitrogen) and transformed and grown as described in the paragraph 4.2.4. Single colonies containing the insert were transferred into LB liquid medium (25g/l, Sigma-Aldrich) with ampicillin and grown overnight at 37^{o}C in the shaking incubator. The plasmid was isolated using GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich) and sequenced using T7 and BGH primers (LGC Genomics). Bacteria containing the plasmids with the insert of the correct sequence and right orientation were transferred into 150 ml LB liquid medium with ampicillin (100 μg/ml, Invitrogen)and grown overnight at 37^{o}C in the shaking incubator. Subsequently the plasmid was isolated by means of EndoFree Plasmid Maxi Kit (Sigma-Aldrich) and once again the sequence was confirmed.

4.2.8 Structural and phylogenetic analysis

The *Sarcr*AKHR amino acid sequence was aligned with AKHR sequences of *M. domestica* (GenBank Acc. No. **XP_005177426.1**), *D. melanogaster* (GenBank Acc. No. **NP_995639.1**) and *G. morsitans* (GenBank Acc. No. **AEH25943.1**) using Clustal Omega (http://www.ebi.ac.uk/

Tools/msa/clustalo/). The transmembrane topology was controlled by the online tool provided by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The putative N-linked glycosylation sites were predicted with theNetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

The phylogenetic analysis of flesh fly AKHR was performed using MEGA 6.1 software. Transmembrane region (TM) 1 – 7TM of all sequences (N- and C-terminal parts of the receptors sequences were removed) was used for an alignment with ClustalW. This alignment was then used for a Maximum-likelihood analysis using the Jones-Taylor-Thornton model (1000-fold bootstrap resampling). The *D. melanogaster* FMRFamide receptor (GenBank Acc. No. **AAF47700.1**) was used to root the tree.

AKHR amino acid sequences used for phylogenetic analysis (Supp. fig. 4.3) derived from *M. domestica*, *D. melanogaster*, *G. morsitans*, *Anopheles gambiae* (GenBank Acc. No. **ABD60146.1**), *Aedes aegypti* (GenBank Acc. No. **CAY77166.1**), *Apis mellifera* (GenBank Acc. No. **NP_001035354.1**), *Nasonia vitripennis* (GenBank Acc. No. **NP_001161243.1**), *B. mori* (GenBank Acc. No. **NP_001037049.1**), *Manduca sexta* (GenBank Acc. No. **ACE00761.1**), *Tribolium castaneum* (GenBank Acc. No. **NP_001076809.1**), *Periplaneta americana* (GenBank Acc. No. **ABB20590.1**), *Blatella germanica* (Gen Bank Acc. No. **ADL60118.1**), *Gryllus bimaculatus* (GenBank Acc. No. **ADZ17179.1**) and *Rhodnius prolixus* (GenBank Acc. No. **AIJ49751.1**). The human gonadotropin-releasing hormone receptor (GenBank Acc. No. **NP_000397.1**) was also included in the analysis.

4.2.9 Cell culture and transfection

Chinese hamster ovary (CHO) WTA11 cells that stably overexpress an apoaequorin, a zeocin resistance gene, and a promiscuous human G protein $G_{\alpha 16}$ were cultured in monolayer in Dulbecco's Modified Eagles Medium nutrient mixture F12-Ham (DMEM/F12, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The cells were cultured in a humid atmosphere with constant supply of 5 % CO_2 at 37^{o}C.

Cell transfections were performed in T75 flasks at 60-80% confluency. Transfection medium was prepared using 3 ml DMEM/F12 medium (without additives) containing 45 µl

Lipofectamine LTX (Invitrogen), 7.5 µg vector construct and 6 µl Plus Reagent (Invitrogen) in 5 ml polystyrene tubes. After an incubation period of 30 minutes at room temperature in basal DMEM/F12, the medium of the cells was removed and the transfection medium was added dropwise to the cells cultures. In addition 3 ml complete medium was added as well. The cells were incubated overnight. An additional 20 ml of complete cell medium was added for the next overnight incubation prior to the cell screen.

4.2.10 Aequorin-luminescence assay

The aequorin-luminescence assay was used to measure calcium release in the transfected CHO WTA11 cells upon ligand exposure. The aequorine is a photoprotein, isolated from the jellyfish *Aequorea aequora* that creates a bioluminescent complex of apoaequorin with the luminophore cofactor, coelenterazine (Shimomura *et al.*, 1962). Apoaequorin calcium ions binding ability causes its oxidation and the aequorin complex decomposes into apoaequorin and carbon dioxide. This goes together with the emission of blue light (λ_{max} = 496 nm) which can be detected by conventional luminometry (Torfs *et al.*, 2002).

CHO WTA11 cell lines are designed to express apoaequorin and therefore they are able to detect an intracellular calcium ion signal. Activation of a GPCR, coupled to a G_q protein increases the intracellular calcium ion concentration by the means of phospholipase C. This means that CHO WTA11 cells are used to check if the transfected receptors acts via a G_q protein and consequently mediates a calcium ion response. CHO-WTA 11 cells also have an extra gene coding for a $G_{16\alpha}$ subunit, which signals via a calcium ion pathway. Additionally, it is a promiscuous G_α, which means it will bind every GPCR. This implies that CHO-WTA 11 cells lines can be used to characterize every kind of GPCR, even if the signaling pathway is unknown (Knight *et al.*, 2003)(Fig. 4.2).

The aequorin-bioluminescence assay was used to measure the calcium release in transfected CHO WTA11 cells upon ligand exposure (as also described in Caers *et al.*, 2015;Verlinden *et al.*, 2015). Transfected cells were detached using 0.2% EDTA-PBS solution and collected in 10 ml DMEM/F12. Subsequently the cell density was determined using the NucleoCounter NC-100 (Chemometic). The cells were centrifuged at 800 rpm for 4 minutes and the pellet was resuspended to a concentration of $5x10^6$ cells/ml in DMEM medium containing 0.1% sterile filtered BSA. Subsequently the whole vial was shielded from the light and 5 µM

Coelenterazine H (Invitrogen) was added. The mixture was gently shaken at room temperature for 4 hours to reconstitute the holoenzyme aequorin. Thirty minutes prior to the screening, the cells were diluted tenfold in 0.1% BSA/DMEM medium.

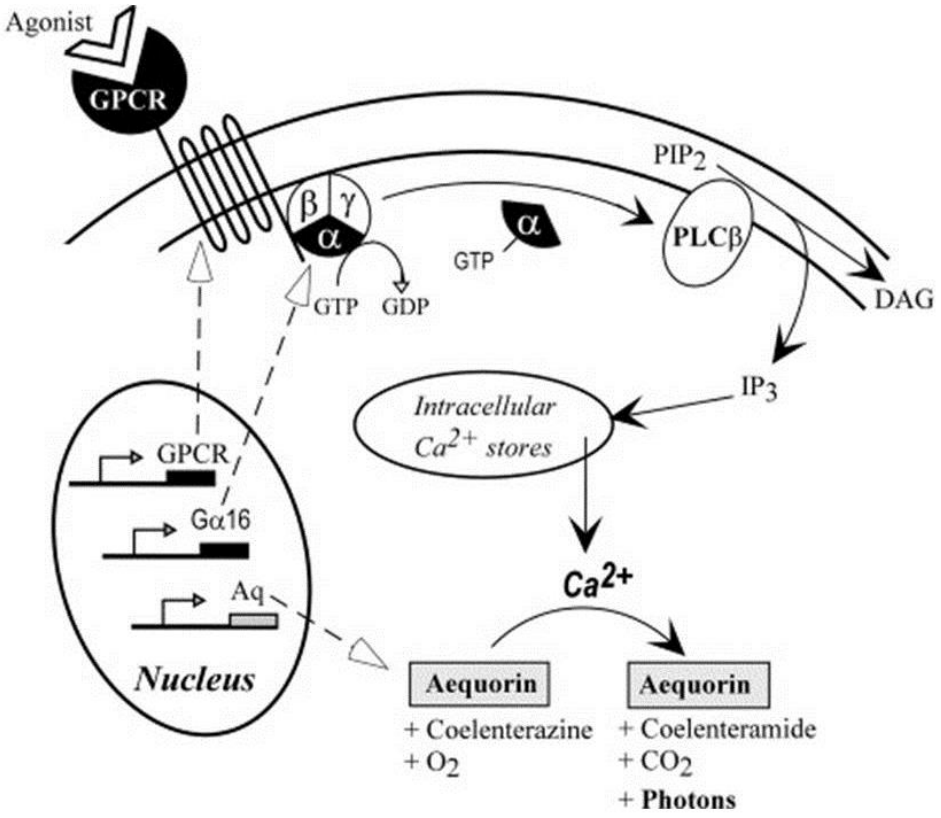


Fig. 4.2 Schematic mechanism of the aequorin-luminescence assay using CHO-WTA 11 cells. The cells stably express apoaequorin and $G_{\alpha16}$. They are transfected with DNA encoding GPCR that is expressed as a transmembrane receptor and binds $G_{\alpha16}$. Upon ligand activation of the receptor, phospholipase C is activated and calcium ions are released from the intracellular stores. The released ions are bound by aequorin resulting in blue light emission. Abbreviations: Aq, apoaequorin; DAG, 1,2-diacylglycerol GDP, guanosine diphosphate; GTP, guanosine triphosphate; GPCR, G protein-coupled receptor; IP_3, inositol triphosphate; PIP_2, phosphatidylinositol biphosphate; PLC, phospholipase C. Image credits: (Knight *et al.*, 2003)

AKH peptide ligands were first dissolved in 30 µl DMSO (due to their hydrophobic nature) and then diluted in 0.1% BSA/DMEM medium into the particular concentrations. Fifty microliters of each of the ligand dilutions were distributed in 96-wells plate, whereas 50 µl of 0.1% BSA/DMEM medium was used as negative control. Fifty microliters of cell solution was injected by the machine in a well per well manner. The calcium response (the light emission) was measured for 30 s using a Mithras LB940 (Berthold Technologies). After that time, 50 µl

of 0.1% Triton X-100 (Sigma) in 0.1 % BSA/DMEM was injected to the cell-ligand mixture and by the cell lysis the total cellular calcium content was released into solution and the signal was measured for another 10 s. The ligand specific response signal was normalized relative to the total signal (ligand and Triton signal, which serves as an indication of the total amount of cells present) using the output file of Microwin2000 (Microtek). Further analysis of three independent duplicate measurements was done in Excel 2010 (Microsoft) and GraphPad Prism 6.

4.2.11 Peptides

Peptide ligands (*Sarcophaga crassipalpis* AKH (identical to *Phote*HrTH (pQLTFSPDWa)), *Glossina morsitans* AKH II (pQLTFSPGWa) and *Tribolium castaneum* AKH II (pQLNFTPNWa)) were synthesized by Pepscan (Nederland). Their purification, as well as concentration determination were identical as described in chapter 3, paragraph 3.2.6.

4.2.12 Statistical analysis

Differences in receptor transcript levels found in the particular tissues were validated by the student's t-test by the comparison of the results of two different feeding conditions.

4.3 Results

4.3.1 Identification and sequence analysis of adipokinetic hormone receptor

The very specific PCR amplification reaction resulted in only one cDNA fragment encoding AKHR (Fig. 4.3) The open reading frame including the stop codon contained 1290 nucleotides (Supp. fig. 4.4) which encoded a 429 amino acids sequence (Fig. 4.4). The calculated molecular weight was 49.363 kDa.

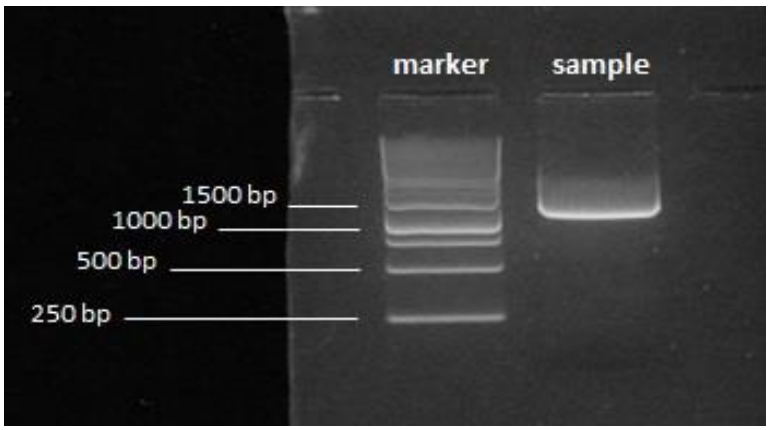


Fig. 4.3 Result of the gel electrophoresis separation of the adipokinetic hormone receptor post PCR amplification. Sample separation shows only one PCR product. The DNA size marker is a 1 kbp DNA ladder (Invitrogen).

Transmembrane topology analysis predicted the expected seven hydrophobic regions creating transmembrane helices (Fig. 4.5) with a probability of 100%. In total 155 amino acids residues were involved in creation of those transmembrane domains. Additionally, the N-terminal end was determined to contain a signal sequence situated outside the cell membrane. The C-terminus was established being located inside the cell. Furthermore, the receptor structure contained three extracellular loops, as well as three intracellular loops. The typical DRY motif (at the end of the third transmembrane domain) and the LXXXNSXXNPXXY motif (in the seventh transmembrane helix) characteristic for all rhodopsin-like GPCR were distinguished. A possible disulfide bridge could be formed between two cysteine residues (C118-C194) localized on the second and third extracellular loops. The possible N-linked glycosylation could be present on N27, N60 and N333 residues.

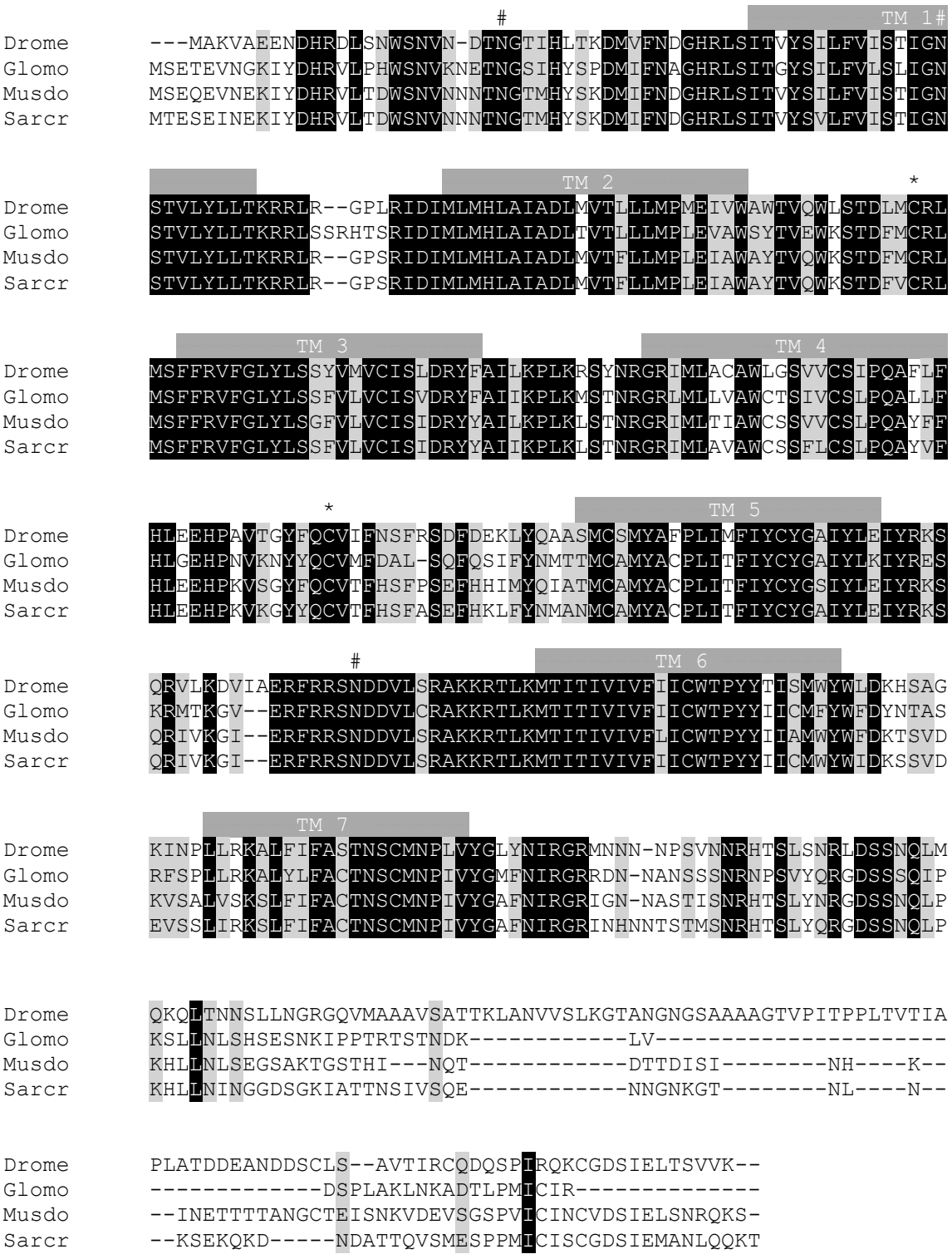


Fig. 4.4 Amino acid sequence alignment of flies' AKHRs. The alignment of *S. crassipalpis* (Sarcr) AKHR against the homologous receptors of *D. melanogaster* (Drome), *G. morsitans* (Glomo) and *M. domestica* (Musdo). The identical amino acid residues between all aligned receptors are marked in black, whereas conserved residues are marked in grey. Predicted transmembrane helixes (TM 1 – 7) are demonstrated in grey bars. The putative N-linked glycosylations are indicated by hashtag, whereas the putative disulfide places are indicated by asterisk.

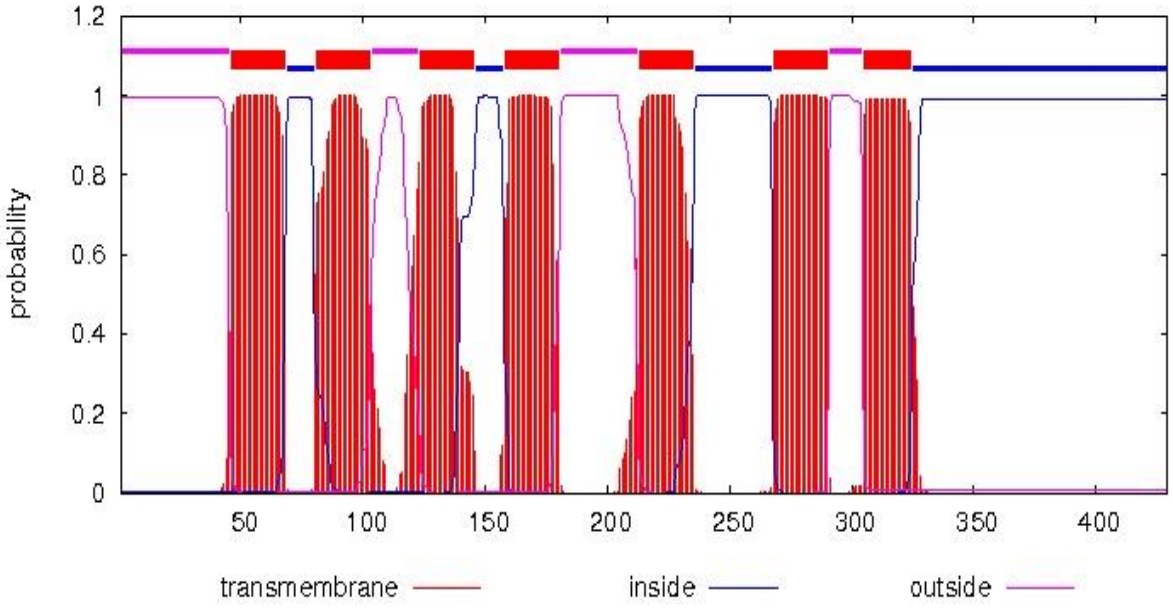


Fig. 4.5 Transmembrane topology analysis of *S. crassipalpis* adipokinetic hormone receptor protein presents seven transmembrane helixes (marked in red), outside and inside regions (marked in pink and blue respectively).

Basic Local Alignment Search Tool searching protein database using a translated nucleotide query (BLASTx, http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis showed the highest similarity (90%) with unfortunately unanotated hypothetical protein FF38_07910 of the blow fly *L. cuprina* (GenBank Acc. No. **KNC20759.1**). Other very high identity (80%) was observed for gonadotropin-releasing hormone receptor of house fly *M. domestica* (GenBank Acc. No. **XP_005177426.1**) as well as (75%) of stable fly *S. calcitrans* (GenBank Acc. No. **XP_013102955.1**). It also resembled AKHR (58%) of other Dipteran species as yellow fever mosquito *A. gambiae* (GenBank Acc. No. **ABD60146.1**) as well as (58%) Hymenoptera honey bee *A. mellifera* (GenBank Acc. No. **NP_001035354.1**) or (52%) Coleoptera red flour beetle *T. castaneum* (GenBank Acc. No. **NP_001076809.1**).

4.3.2 Phylogenetic analysis

Phylogenetic analysis (Fig. 4.6) clearly showed that all flies' AKHR sequences cluster together. Similar to the BLAST search, the closest relative to *S. crassipalpis* AKHR was the receptor sequence of *M. domestica*. Although the sequences of the AKHRs of the major insect orders (Hymenoptera, Coleoptera, Orthoptera, Lepidoptera, Hemiptera) formed

separate clusters, the phylogenetic relationships between the different groups was not always clear. This was also reflected in some of the rather low bootstrap values.

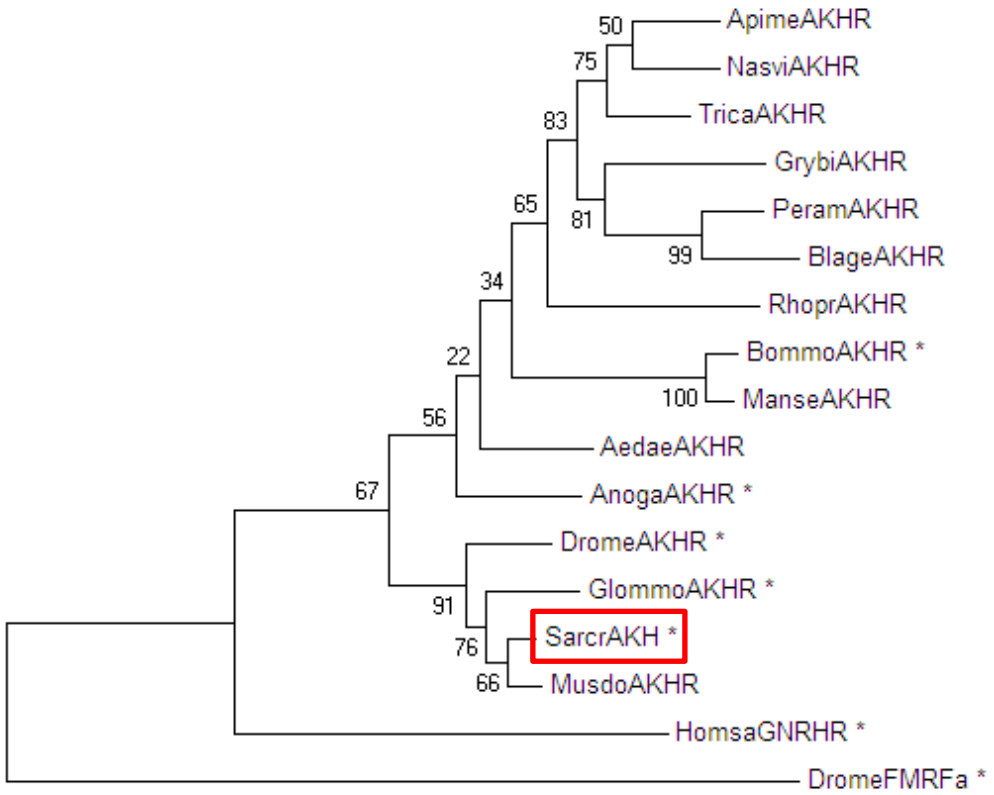


Fig. 4.6 A maximum likelihood phylogenetic tree of adipokinetic hormone receptors and human gonadotrophin-releasing hormone receptor. Bootstrap values based on 1000 replicates are indicated on the tree nodes. The *D. melanogaster* FMRFa receptor was used to root the tree. Abbreviations used: Aedae, *Aedes aegypti*; Anoga, *Anopheles gambiae*; Apime, *Apis mellifera*; Blage, *Blatella germanica*; Bommo, *Bombyx mori*; Drome, *Drosophila melanogaster*; Glomo, *Glossina morsitans*; Grybi, *Gryllus bimaculatus*; Homsa, *Homo sapiens*; Manse, *Manduca sexta*; Musdo, *Musca domestica;* Nasvi, *Nasonia vitripennis*; Peram, *Periplaneta americana*; Rhopr, *Rhodius prolixus*; Sarcr, *Sarcophaga crassipalpis*; Trica, *Tribolium castaneum*.

4.3.3 Receptor transcript level and tissue distribution analysis

Based on the observed quantitative differences of AKH peptide in CC of observed between sugar and liver-fed female flies (described in the previous chapter), the receptor transcript tissue distribution analysis was also performed on sugar and protein-fed (5 hours post protein feeding) flies (Fig.4.7).

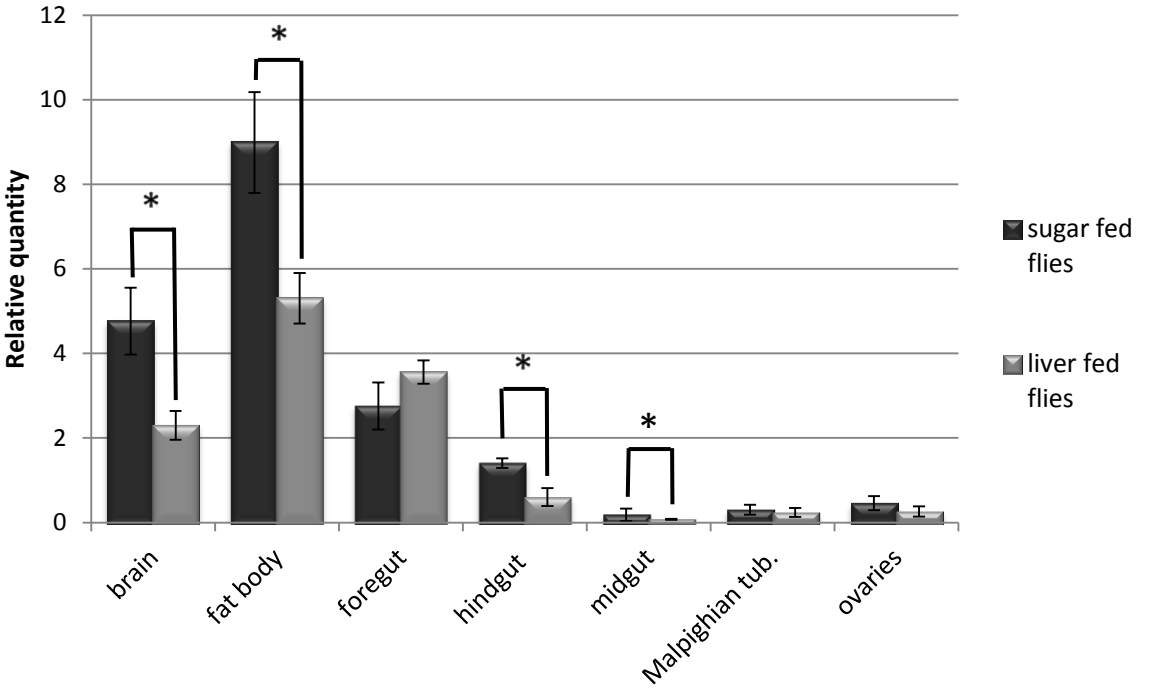


Fig. 4.7 Tissue distribution profile of adipokinetic hormone receptor. Quantification of the receptor transcript level in seven different tissues of sugar and liver fed adult female flesh flies. Significant differences ($p<0.05$) are indicated by asterisk (three biological replicates of ten pooled tissues each); mean *± SD.*

In general, AKHR gene expression was obviously the most prominent in the fat body (in sugar as well as in liver fed conditions). AKHR transcripts were also highly abundant in the brain, foregut and hindgut. Trace amounts of the receptor transcript were observed in the ovaries, Malpighian tubules and midgut.

Significant differences of AKHR transcript levels between two feeding conditions were measured in the fat body, brain, hindgut and midgut ($p_{fat\ body}=0.018$; $p_{brain}=0.020$; $p_{hindgut}=0.018$; $p_{midgut}=0.009$) of liver-fed females.

4.3.4 Functional activation and dose response analysis of S. crassipalpis *adipokinetic hormone receptor*

S. crassipalpis AKHR was expressed in CHO-WTA11 cells containing a promiscuous $G_{\alpha16}$ subunit. The receptor dose response activation was tested using three AKH peptides derived of two dipteran species, *Sarcophaga crassipalpis* and *Glossina morsitans* and one coleopteran species of *Tribolium castaneum*. Both dipteran AKH peptides, which differ in one

amino acid localized on the seventh position (aspartic acid and glycine respectively), gave very similar results and clearly elicited a dose-dependent response of an intracellular Ca^{2+} increase with EC_{50} value of 20.82 and EC_{50} value of 44.91 nanomolar respectively (Fig.4.8).

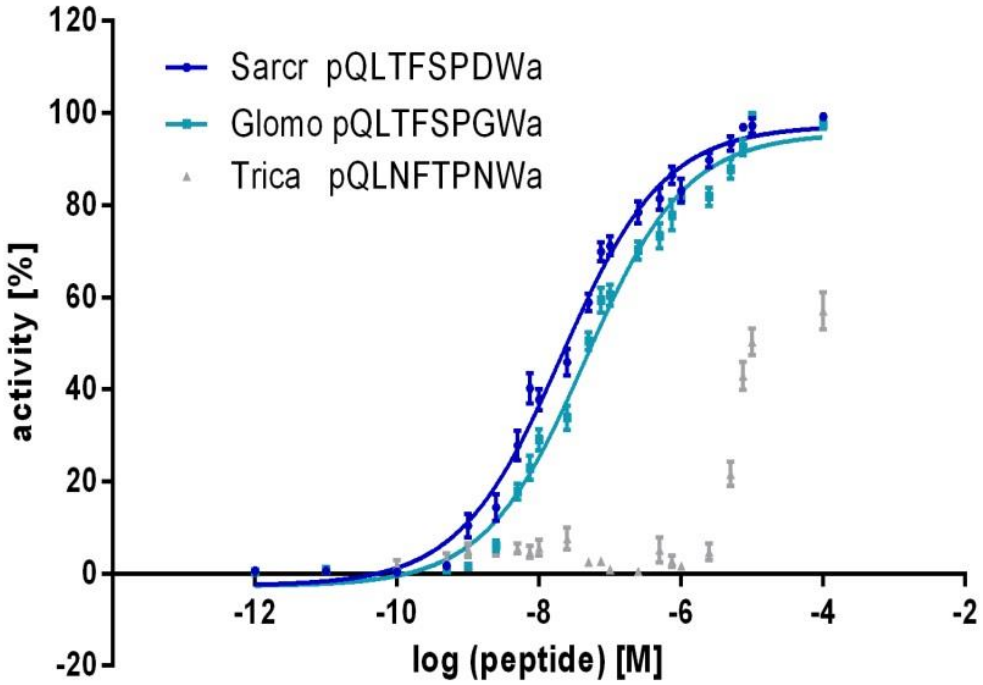


Fig. 4.8 Dose response curve for bioluminescence responses induced by *Sarcophaga crassipalpis* AKH (Sarcr), *Glossina morsitans* AKH II (Glomo) and *Tribolium castaneum* AKH II (Trica) in CHO-WTA11 cells transfected with *S. crassipalpis* AKHR (three independent biological replicates); mean *± SEM.*

Maximal response of the receptor was achieved with concentration of ten micromolar or higher. No response could be measured for 0.1 nanomolar solutions for both flies' AKHs. *Trica*AKHII could also activate the receptor although much higher concentrations were needed (EC_{50} value could not be determined since no plateau was reached with the used peptide dilutions. Higher ligand concentrations could not be dissolved properly in low, non-harmful for the cells, percentage of DMSO).

No Signal was observed for CHO-WTA11 cells transfected with empty pcDNA3.1/V5-His-TOPO TA expression vector construct.

4.4 Discussion

Sarcophaga crassipalpis AKHR is a glycoprotein hormone receptor containing seven transmembrane helices characteristic for the family of G protein-coupled receptors. It also includes the several specific amino acids motifs typical for their A class of rhodopsin-like receptors (Costanzi, 2012). The possible disulfide bridge formation between cysteines located on the extracellular loops is helping in creating and maintaining of the ligand binding pocket structure (Miller & Gao, 2008;Zels *et al.*, 2014). Insect AKHRs and the vertebrate gonadotropin-releasing hormone receptors are structurally and evolutionary related (Hauser *et al.*, 1998;Lindemans *et al.*, 2011).

Among flies, all AKHR amino acid sequences are very well conserved starting from the beginning up to about 35 amino acids behind the last transmembrane domain. Larger scale alignment containing other insect species derived AKHR sequences is indicating overall evolutionary conservation.

Based on the insects' phylogenetic analysis using the protein coding genes of species spanning different orders, the Diptera order consists of flies and mosquitoes forming two groups derived from a common ancestor (http://www.livescience.com/48663-insect-family-tree-evolution.html) (Fig.4.9).

Surprisingly, not all dipteran AKHRs cluster together in our phylogenetic tree, which is similar to the Attardo (2012) phylogenetic analysis. The members of the main orders in our analysis do cluster accordingly, but also the relative position of the different orders is not in agreement with the insect family tree. First of all more sequences from different orders may shed a better light on this, but it may also be that convergent evolution between different insect orders for this specific gene has occurred due to e.g. similar energy needs caused by similar environments.

The big dissimilarities observed between flies' and mosquitoes' AKHR amino acid sequences excluded mosquitoes from the PCR primer design assignment.

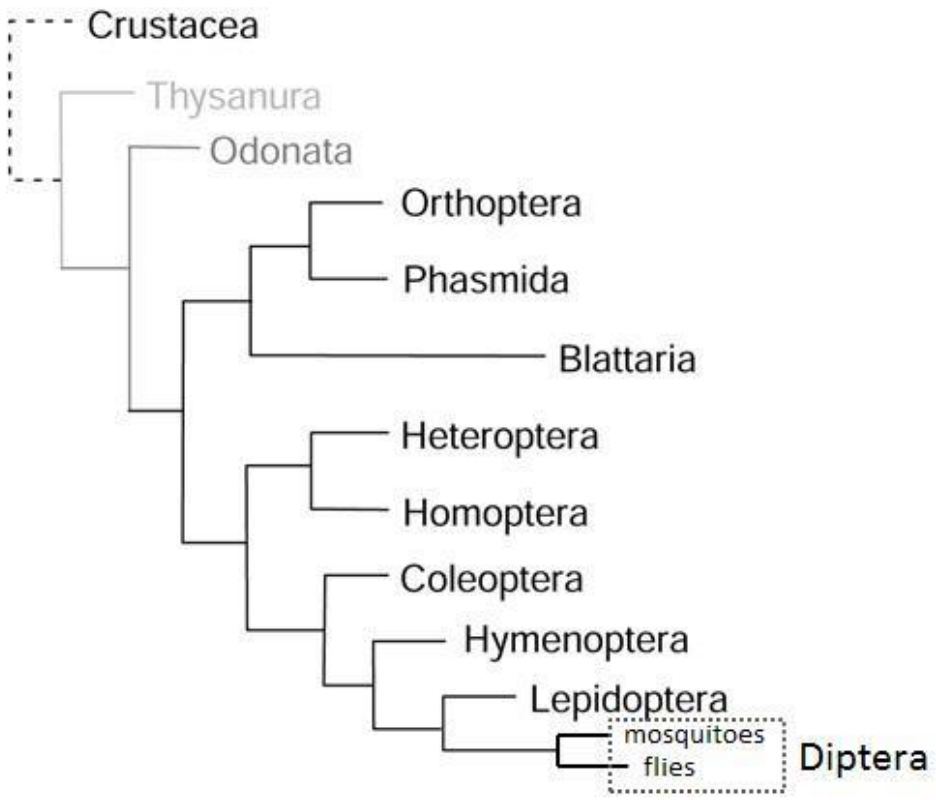


Fig. 4.9 Simplified phylogenetic analysis of the Insecta class representing the evolutionary relationships among different insect orders and structure of Diptera order. Crustacea are used as an arthropod outgroup. Light gray, dark gray and black are indicating respectively Archaeognatha (primitive wingless insects), Paleoptera (primitive winged insects); Neoptera (higher insects). Image credits: http://www.genomebiology.com/2003/4/3/107/figure/F1

The highest AKHR transcript levels in *S. crassipalpis* were measured in the fat body. This corresponds with the main and also best known function of AKH, lipid and carbohydrate mobilization from the fat body during high energetic processes such as flying (Van der Horst *et al.*, 2003). Also the brain contains high AKHR transcript levels, which is similar as observed in the silkworm, *B. mori* (Shi *et al.*, 2011). As was also observed in the cockroach *P. americana* high AKHR transcript levels were detected in the initial part of the digestion tract (Bodláková *et al.*, 2016). These authors demonstrated that AKH was indeed involved in the regulation of digestive enzymes. Similarly, AKH was demonstrated to be involved in moving carbohydrates up into the midgut for digestion in the anautogenous black blow fly *Phormia regina* (Stoffolano *et al.*, 2014), which also points out the presence of the AKHR in the foregut of this fly.

There is almost no AKHR transcript present in the midgut tissue. This explains the impossibility of proteolytic activity stimulation using midguts incubated *in vitro* in physiological mediums including different AKH doses. Additional supplementation of this *in vitro* midgut culture with fat body, brain, foregut or hindgut (all these tissues express the receptor) did not work either. A retrograde action of AKH towards the brain can be excluded as AKH stimulates midgut proteolytic activity in sugar-fed and liver-primed decapitated flies. Altogether our experiments suggest that AKH stimulates digestive activity *in vivo* in some indirect and much more complex way.

The decrease of the AKHR transcript levels upon a protein meal is not only observed in liver-fed *S. crassipalpis*, but also in blood-fed *A. gambiae* (Kaufmann & Brown, 2006). It can be related to the feeding and digestion processes that provide a lot of different energy carrying molecules passing into the hemolymph which should be consumed first. The lower abundance of AKHR prevents the use of stored reserves and preserves them for future activities or for the 'emergency' case when no food is available. Knockdown of the AKHR in the cricket, *G. bimaculatus*, resulted in decreased levels of energy carrying molecules in the hemolymph and increased levels of triacylglycerol in the fat body. As expected, this also led to an increase of the feeding frequency of that insect (Konuma *et al.*, 2012). Furthermore, post liver feeding decrease of the AKHR gene expression level corresponds well with the changes of the AKH peptide observed between sugar and liver-fed flesh flies.. High expression of the particular receptor, while small amount of the available interacting ligand is available, is also unfavorable for cells. Nonetheless to verify all these hypothesis, further research is needed.

The pharmacological results of the *Sarcr*AKHR correspond well with other pharmacological studies of AKHRs. The one amino acid difference (aspartate-glycine difference at the seventh residue) between the two dipteran AKH peptides does not seem to make any difference for receptor activation as observed in *D. melanogaster*, where the synthetic ligand having the alanine-substitution on this position elicits almost the same receptor activation response as observed for the original peptide ligand (Caers *et al.*, 2012). In both flies' ligands the entire essential N-terminal pentapeptide (Gäde & Hayes, 1995) remains identical which guarantees high binding affinity. Coleopteran *T. castaneum* AKH II peptide differs much more in its sequence compared to the previous ones. This is also reflected in its receptor activation capability. *Trica*AKH II has an amino acid substitution on the third position (threonine into

asparagine) and also on the fifth position (serine into threonine) additionally to an amino acid substitution on the seventh position (aspartate or glycine to asparagine). These residues seemingly play an important role in ligand-receptor binding and activation as demonstrated in *D. melanogaster* and *A. gambiae* (Caers *et al.*, 2012). Nevertheless, both substitutions (at the third and fifth position) seen in *Trica*AKH II represent changes of one amino acid with the polar uncharged side chain for the other one with the same properties. They still allow this peptide to bind and activate the receptor although much higher concentrations are needed. Substitutions by amino acids with different nature are described to almost completely abolish interaction with AKH receptors (Caers *et al.*, 2012;Caers *et al.*, 2015).

CHAPTER 5.

Pharmacological regulation of digestion and ovarian development in the anautogenous flesh fly, *Sarcophaga crassipalpis*, by injection of 6-hydroxydopamine‡

‡ Parts of this chapter are published in: Bil,M.; Huybrechts,R. (2016) Pharmacological regulation of digestion in the anautogenous flesh fly, Sarcophaga crassipalpis, by simple injection of 6-hydroxydopamine. Archives of Insect Biochemistry and Physiology 91, 137-151.

5.1 Introduction

In adult anautogenous female flesh flies, the digestion of the protein meal starts a cascade of events; of which the most important is the synthesis of yolk proteins (YPs) by the fat body and follicle cells (Huybrechts & De Loof, 1977;Huybrechts & De Loof, 1982). In the previtellogenic period, the fat body cells become competent for large scale production of YPs. The induction of this competency is not only regulated by the food-derived energy and building blocks, but is also dependent on juvenile hormone (Borovsky *et al.*, 1985;Raikhel *et al.*, 2002). In mosquitoes, protein meal activation stimulates brain cells to produce an ovary ecdysteroidogenic hormone (OEH), a female gonadotropin (Brown *et al.*, 1998). In spite of available EST (Hahn *et al.*, 2009) and genomic data (Vicoso & Bachtrog, 2015;Anstead *et al.*, 2015) an orthologue of this OEH in Sarcophagidae or Calliphoridae remains untraceable. Nevertheless, following a protein meal, the ovaries of both mosquitoes and flies release an ecdysone hormone (Hagedorn *et al.*, 1979) that is later hydroxylated to 20-hydroxyecdysone. It directly stimulates the fat body for the YPs production and secretion into the hemolymph, from where they are targeted and sequestrated by the developing oocytes (Huybrechts & De Loof, 1977;Huybrechts & De Loof, 1982;Briers & Huybrechts, 1984;Raikhel *et al.*, 2002;Attardo *et al.*, 2005).

Six-hydroxydopamine (6-OHDA), the hydroxylated form of dopamine, also known as oxidopamine, is a catecholaminergic neurotoxin that in vertebrate and invertebrate model organisms is frequently used to induce Parkinson's disease (Dawson *et al.*, 2010;Liu *et al.*, 2011). Oxidopamine (6-OHDA) enters axons via the dopamine reuptake mechanism using both the dopamine (Shimada *et al.*, 1991;Usdin *et al.*, 1991) and the adrenaline transporters (Pacholczyk *et al.*, 1991).

It has two independent modes of action. The main mechanism of its neurotoxicity is oxidative stress (Fig. 5.1). Because 6-OHDA is a very unstable molecule in the pH of the cytoplasm, it auto-oxidates and produces a lot of reactive oxygen species (hydrogen peroxide, hydroxyl radical, superoxide anions and reactive quinones) that can covalently bind the nucleophilic groups of other vital molecules, such as –SH, -NH_2 and phenolic OH group, and cause their denaturation (reviewed by Sachs & Jonsson, 1975). Oxidopamine (6-OHDA) is a perfect substrate for monoamine oxidase, it significantly increases the level of hydrogen peroxide and destroys the neurons (reviewed by Blum *et al.*, 2001). Nonetheless, it is possible to limit the destructive effect to only dopaminergic neurons by the use of desipramine, which blocks 6-OHDA uptake by adrenergic transporters (reviewed by Sachs & Jonsson, 1975;Zigmond *et al.*, 1992). Another way of action is by direct inhibition of the respiratory enzymes of the mitochondrial respiratory chain at the level of complex I. As a result, intracellular ATP production is compromised, causing neural cell death (Glinka *et al.*, 1997;Rodriguez-Pallares *et al.*, 2007). Additionally, 6-OHDA is also suspected to block the α-adrenergic receptors but this single report has not been confirmed (Nakamura & Thoenen, 1971).

Oxidopamine (6-OHDA) effects have been studied in different insects. Initially, it showed to be a functional neurotoxin that affect molting and duration of the last larval instar in the locust, *Locusta migratioria*, the greater wax moth, *Galleria mellonella* and in the house cricket, *Acheta domestica* (Hiripi & Rózska, 1980;Warton, 1981;Rózsa *et al.*, 1986). In the anautogenous flesh fly *Neobellieria bullata*, formerly *Sarcophaga bullata*, Huybrechts previously demonstrated inhibition of the vitellogenin synthesis and ovarian development, following the liver feeding proceeded injection of a pharmacological dose of 6-OHDA

(Huybrechts, 1982). In the autogenous housefly, *Musca domestica,* 6-OHDA similarly retarded ovarian development (Gerst & Adams, 1989).

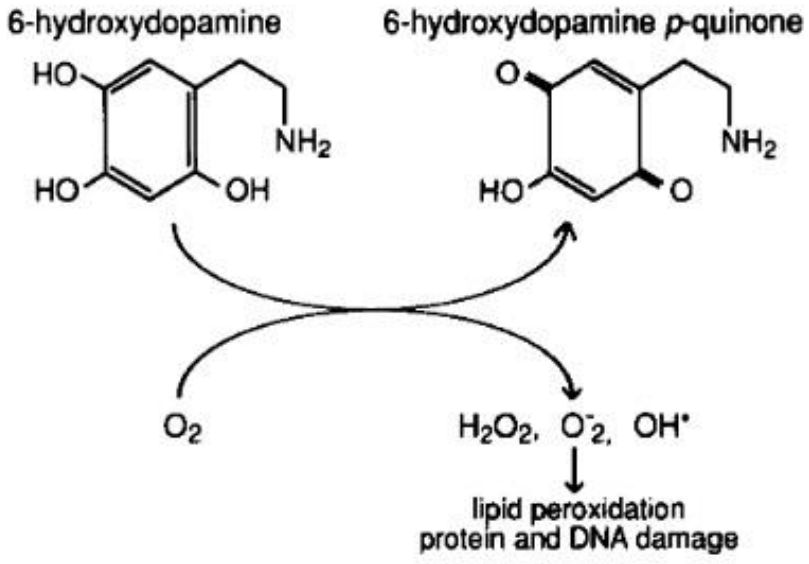


Fig. 5.1 The auto-oxidation action of 6-OHDA. Cytoplasmic auto-oxidation results in production of a lot of reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, superoxide anions and reactive quinones. Image credits: (Zigmond *et al.*, 1992)

In the previous chapters, I explained the proteolytic digestion process and its control in the anautogenous female flesh fly, *Sarcophaga crassipalpis*. In this last experimental chapter, I presented 6-OHDA as a potential research tool that could induce similar effects to fly decapitation. Similar to early decapitation, an early 6-OHDA injection was able to inhibit proteolytic digestion in liver-fed flies, whereas late, postponed treatment had no more effect on enzymatic activity. Additionally, the inhibition of digestion by 6-OHDA showed a dose-dependent nature and hitherto, digestive proteolytic activity could be down-regulated to the desired level by the use of different 6-OHDA doses. This pharmacological decapitation tool also allowed us to demonstrate a tight relationship between the amount of food derived proteins absorbed into the hemolymph (from ingested food) and the capability of the ovaries to develop within the context of a food-dependent reproductive strategy.

5.2 Materials and methods

5.2.1 Insect rearing

Flies used for experiments were reared as described in chapter 2, paragraph 2.2.1.

5.2.2 6-hydroxydopamine treatment

Using a Hamilton microsyringe, each carbon dioxide-anesthetized fly was injected intra-abdominally with 1 μl of the particular, dependent on experiment dose of 6-OHDA (Sigma-Aldrich), freshly dissolved in flesh fly Ringer's solution. After injection, flies were placed in small separate cages for 15 min to recover and to avoid changes in behavior caused by the stress of anesthesia or injection.

In further rescue experiments, flies were additionally injected with 1 μl of different doses (1 ng; 10 ng; 100 ng; 1 μg and 10 μg) of dopamine (Sigma-Aldrich), L-DOPA (Sigma-Aldrich), adrenaline (Sigma-Aldrich) and noradrenaline (Sigma-Aldrich). The additional injection took place immediately after liver feeding.

5.2.3 Feeding and decapitation procedures

Strict feeding condition and animal decapitation were performed as described in chapter 2, paragraph 2.2.4.

5.2.4 Midgut dissection

The tissue dissection procedure was the same as described in chapter 2, paragraph 2.2.2.

5.2.5 Determination of midgut proteolytic activity

Digestive proteolytic activity was measured as described in chapter 2, paragraph 2.2.5.

5.2.6 RNA isolation and cDNA synthesis

RNA was isolated from the midgut tissue and reverse transcribed as described in chapter 2, paragraph 2.2.6.

5.2.7 Quantification of trypsin gene expression in 6-hydroxydopamine treated flies

Trypsin gene transcript level (Hahn transcriptome: **Hahn.Fly.10868.C3**, established as the most abundant early trypsin gene found in liver-fed flies) was measured using the quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) as described in chapter 3, paragraph 3.2.6.

5.2.8 Toxicity effect of 6-hydroxydopamine

Flies were divided into three groups of 50 insects, once injected with different doses (0 μg; 20 μg; 40 μg; 60 μg; 80 μg and 100 μg corresponding to 0 mM; 100 mM; 200 mM; 300 mM; 400 mM and 500 mM) of 6-OHDA. Subsequently they were placed in small observation cages containing sugar and water. These cages were located in the breading room to ensure the optimal rearing conditions. The number of dead flies was counted every day, for 4 days around 12 PM.

5.2.9 Development of ovaries under 6-hydroxydopamine treatment

Flies were divided into seven groups, once injected with different doses (0 μg; 20 μg; 40 μg; 60 μg; 80 μg and 100 μg corresponding to 0 mM; 100 mM; 200 mM; 300 mM; 400 mM and 500 mM) of 6-OHDA (50 flies in each group). Afterwards, all flies were offered sugar, water and a piece of liver. The development of the ovaries was checked on 1, 2 and 4 day/s after the 6-OHDA treatment and was determined by the measurement of the diameter of each ovary using a caliper. Ten flies from each group were checked on each particular day. Photos were taken using an AmScope SM-4TZ-144A professional tri-ocular Stereo Zoom Microscope equipped with digital camera (AmScope MU035).

5.2.10 Ovarian development under different nutritional manipulations

Fly ovarian development was investigated under three different dietary conditions: complex protein meal of a cow liver; artificial protein meal of 500 mg BSA (Sigma-Aldrich), dissolved in 2 ml Ringer's solution, in order to provide salts; or 3 ml amino acid solution of IPL-41 Insect Medium (Sigma-Aldrich). Insects had constant access to the food for the whole duration of the experiment.

Ovarian development was also tested in sugar-fed female flies, that were intra-abdominally injected with 1 μl of 1 μM solution containing all essential amino acids (Sigma-Aldrich) dissolved in *S. crassipalpis* Ringer's solution, which contained a trace amount of acetonitrile to dissolve hydrophobic molecules.

Four days old sugar-fed flies were used as a starting point for all experimental treatments.

5.2.11 Statistical analysis

Statistical analysis of midgut proteolytic activity between different groups of flies was tested as described in chapter 2, paragraph 2.2.8.

5.3 Results

5.3.1 6-hydroxydopamine mimics decapitation

Female flies were divided into three experimental groups: two groups injected with 1 μl of Ringer's solution (positive and negative controls) and one group injected with 1 μl Ringer's solution containing 100 μg (0.5 mM) 6-OHDA. After 15 minutes of recovery, all flies were attracted to and fed on a piece of cow liver to stimulate the proteolytic activity in their midgut. Additionally, flies of the negative control group were immediately decapitated after protein feeding.

Midgut proteolytic activity of decapitated liver-fed flies (negative control) did not increase. The digestive activity profile of 6-OHDA-treated intact flies also did not increase and the measured profile almost completely overlapped with the enzymatic profile of the negative control flies ($p_{0\text{-}6h}$=1) (Fig. 5.2).

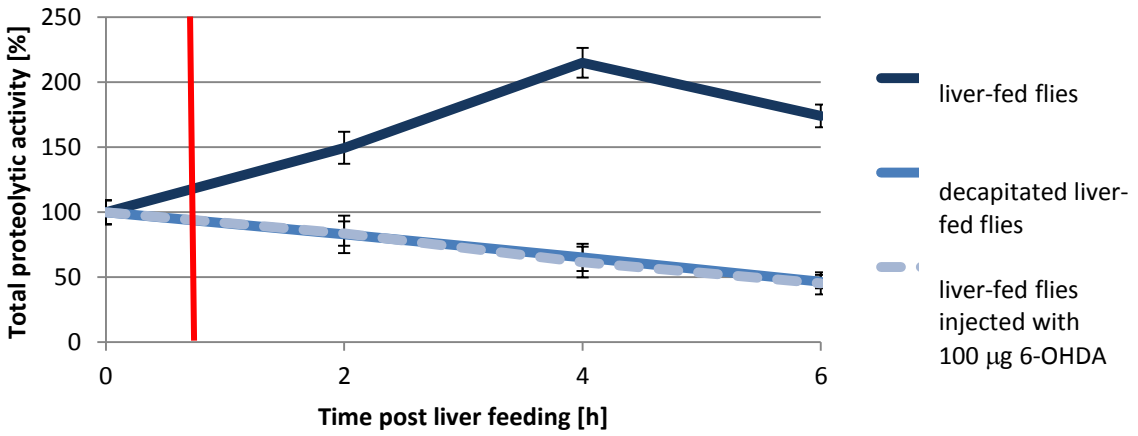


Fig. 5.2 Midgut proteolytic activity profile of liver-fed flies, decapitated liver-fed flies and liver-fed flies injected with 100µg (0.5 M) 6-OHDA. Insects were injected 15 minutes before the beginning of the liver feeding. Enzymatic activity profile of 6-OHDA treated-flies is similar to the digestive activity profile observed in decapitated liver-fed flies. Red line indicates the end of feeding and decapitation moment (four biological replicates of three pooled guts each); mean *± SD*.

5.3.2 6-hydroxydopamine as a regulator of digestion

To further characterize the nature of the digestion-inhibiting function of 6-OHDA and its capacity to prevent the increase in enzymatic activity, induced by liver feeding, flies were divided into six groups treated with a 1 µl injection of different amounts of 6-OHDA (0 µg, 20 µg, 40 µg, 60 µg, 80 µg, 100 µg corresponding to 0 mM; 100 mM; 200 mM; 300 mM; 400 mM and 500 mM). After a recovery period, all fly groups were fed with liver. Midgut samples were collected at 4 hours ppf (at the moment of the highest proteolytic activity observed in intact liver-fed flies).

The results clearly showed an inverse correlation between the increasing dosage of 6-OHDA and prevention of the increase in digestive activity that is normally seen post liver feeding (Fig. 5.3A). There are significant differences between flies injected with 0 µg versus flies injected with 20 µg, 40 µg, 60 µg, 80 µg and 100 µg of 6-OHDA ($p_{0vs20;40;60;80;100}$=0.0072; 0.0016; 0.0004; 0.0000). The analysis of the basic digestive activity of sugar-fed flies and 6-OHDA treated liver-fed flies proved that the injection of 60 µg 6-OHDA resulted in spite of the liver feeding in a proteolytic activity similar to that observed in non-protein-fed flies (p=0.3782) (Fig. 5.3B).

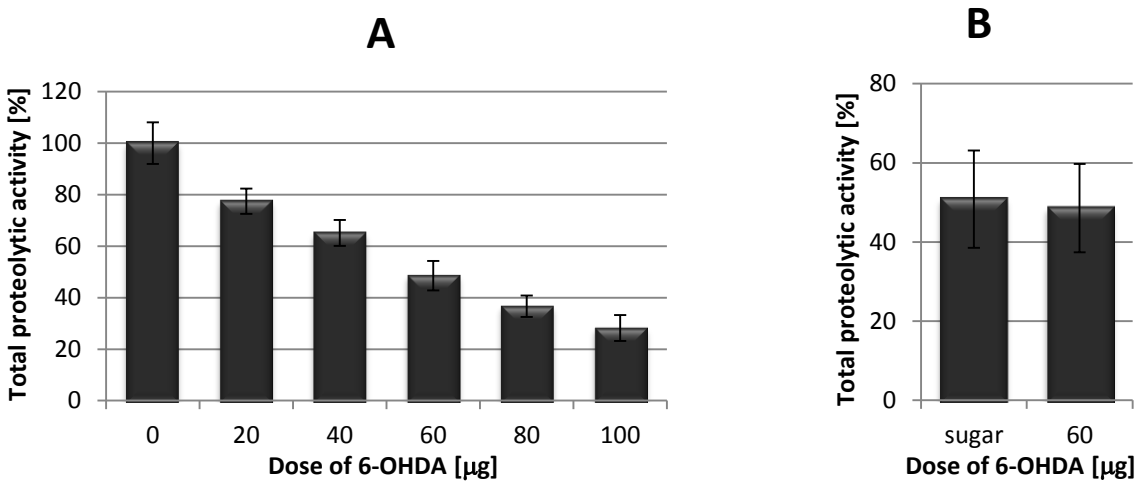


Fig. 5.3 Oxidopamine (6-OHDA) dose-dependent inhibition of midgut proteolytic activity measured at 4 hours ppf (A). Increasing injected dose of 6-OHDA results in stronger inhibition of digestive activity in liver-fed flies. The comparison of the enzymatic activity of sugar-fed flies and 60 µg 6-OHDA injected liver-fed flies measured at 4 hours ppf (B). Dose of 60 µg 6-OHDA prevents the increase in enzymatic activity, as normally seen in liver-fed flies, and the level did not exceed the digestion level observed in sugar-fed flies. All injection took place 15 minutes before liver feeding (four biological replicates of three pooled guts each); mean ± *SD*.

Earlier experiments performed in chapter 2 demonstrated that delayed decapitation (executed later than 4 hours post liver feeding), did no longer prevent the activation of the digestion process. For this reason, we also tested the effect of a 6-OHDA injection into liver-fed flies, during their early and late stages of proteolytic digestion initiation (before and after 4 hour ppf).

Similar to the early decapitation effect, an early injection (2 hours ppf) of 100 µg 6-OHDA could stop the increase of enzymatic activity in liver-fed insects (Fig. 5.4). Nevertheless, a later injection (6 hours ppf) of the same dose, similar to the late decapitation manipulation, no longer had a negative influence on the digestion process. Proteolytic activity of late injected liver-fed flies looked similar to the enzymatic activity measured in non-treated liver-fed flies (p_{0h}=1.000000; p_{2h}=1.000000; p_{4h}=0.980446; p_{6h}=0.999217; p_{8h}=0.999987; p_{10h}=1.000000).

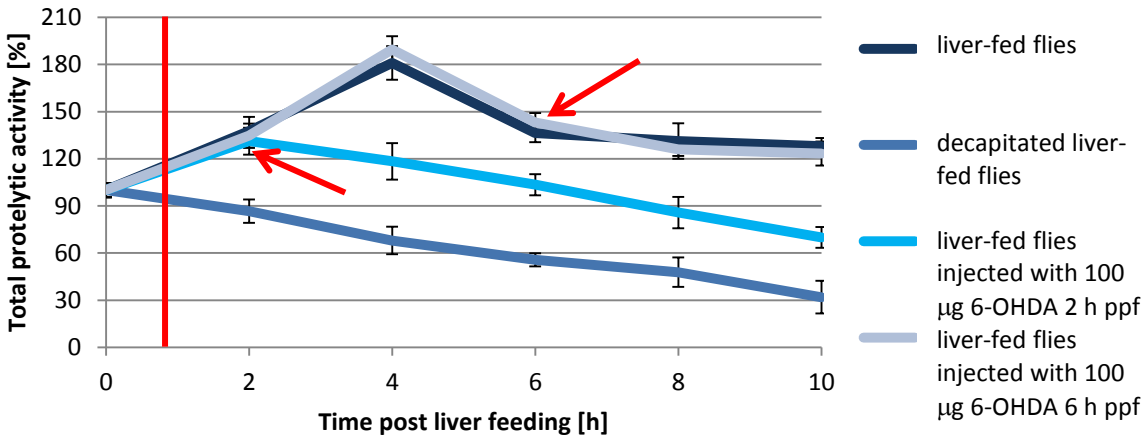


Fig. 5.4 Midgut proteolytic activity of liver-fed flies, decapitated liver-fed flies, liver-fed flies injected with 100 µg 6-OHDA at 2 or 6 hours ppf. Early injection can inhibit enzymatic activity in liver-fed flies, in contrast to late injection that does not have any influence on digestion any more. Red line indicates the end of feeding and decapitation (if necessary) moment, whereas the red arrows indicate injection moments (four biological replicates of three pooled guts each); mean ±*S*D.

5.3.3 6-hydroxydopamine regulates trypsin gene expression at post-transcriptional level

In order to check if the trypsin gene expression was down-regulated on a transcriptional or translational level (possible inhibitory mechanism of 6-OHDA), flies were again divided into three groups and respectively treated with 1 µl of Ringer's solution (liver-fed flies of positive control and sugar-fed flies of negative control group) or 1 µl Ringer's solution containing 100 µg 6-OHDA. After the recovery time, all insects were fed with liver or sugar respectively.

The gene transcript level of the most abundant trypsin found in liver-fed flies, which was established to encode an early trypsin, showed a clear increase (compared to sugar-fed flies) in both liver-fed and liver-fed 6-OHDA-injected flies (Fig. 5.5). Trypsin gene expression of oxidopamine-treated insects seemed to increase a little bit less than observed in the positive control of liver-fed flies, nevertheless, at 4 hours ppf, it constituted 204% of the transcript level measured in sugar-fed flies. Additionally, there was no significant difference between liver-fed and liver-fed 6-OHDA-injected flies, measured at 2 and 8 hours ppf (p_{2h}=0.989572; p_{8h}=0.976447). Moreover, the significant difference was measured at every tested time point between the gene transcript levels of sugar-fed and liver-fed 6-OHDA-injected flies.

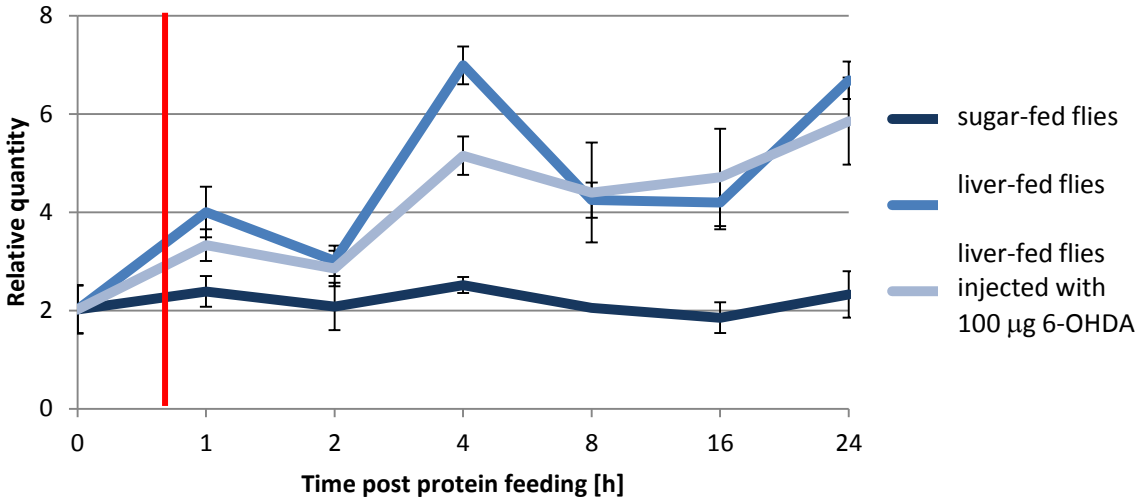


Fig. 5.5 Trypsin gene expression in sugar-fed, liver-fed and liver-fed 100 µg (0.5 M) 6-OHDA injected flies measured up to 24 hours ppf. Compared to the sugar-fed flies, trypsin transcript level increased in both liver-fed conditions. All insects were injected with Ringer's solution or 6-OHDA 15 minutes before feeding. Red line indicates the end of feeding (four biological replicates of three pooled guts each); mean *± SD*.

5.3.4 Attempt to rescue the impaired midgut proteolytic activity process

In order to check if the 6-OHDA digestion inhibition was a reversible process, flies were first injected with 100 µg 6-OHDA, then after short recovery time fed with liver and subsequently injected with a different doses (1 ng; 10 ng; 100 ng; 1 µg and 10 µg corresponding to 5.3 µM; 52.7 µM; 0.5mM; 5.3 mM and 52.7 mM) of dopamine. The proteolytic activity measured after 4 hours ppf indicated that none of the tested dopamine doses was able to rescue the digestion suppressing effect of 6-OHDA (Fig. 5.6).

The same negative results were obtained by trying to rescue the impaired midgut proteolytic activity using L-DOPA, adrenaline and noradrenaline (data not shown).

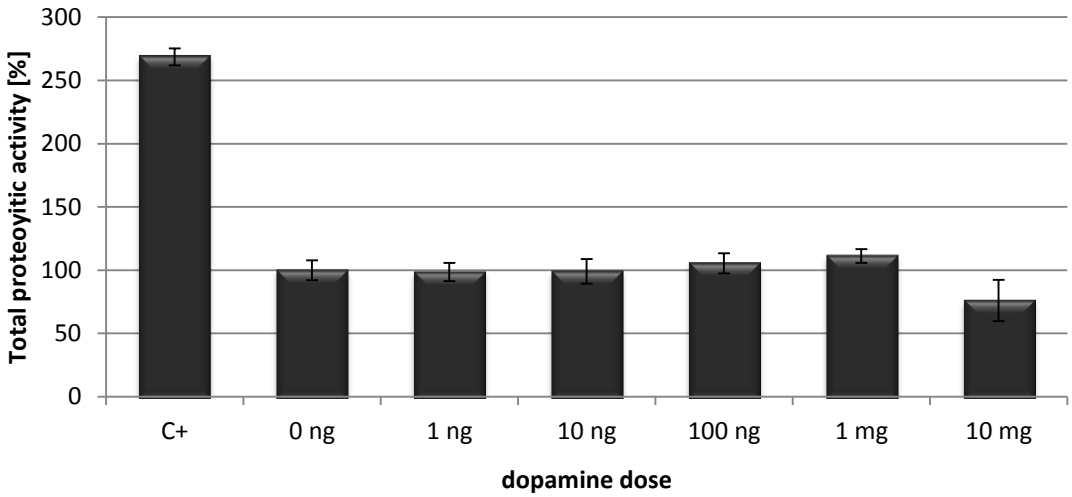


Fig. 5.6 Midgut proteolytic activity of flies treated with 100 μg (0.5 M) 6-OHDA prior liver feeding and an additional injection of different doses of dopamine (post liver feeding) measured 4 hours ppf. None of the tested dose is able to rescue the 6-OHDA inhibited process. Positive control sample (C+) represented non-treated liver-fed flies (four biological replicates of three pooled guts each); mean *± SD*.

The same negative results were obtained by trying to rescue the impaired midgut proteolytic activity using L-DOPA, adrenaline and noradrenaline (data not shown).

5.3.5 Dose-dependent toxicity effect of 6-hydroxydopamine

To evaluate the toxicity effect of 6-OHDA in treated flies, 4 days old sugar-fed females were injected with different concentrations (100 μg; 80 μg; 60 μg; 40 μg; 20 μg and 0 μg corresponding to 500 mM; 400 mM; 300 mM; 200 mM; 100 m M and 0 mM) of 6-OHDA and their mortality was checked for four subsequent days (Fig. 5.7 and Table 5.1).

On the first day post injection, there was almost no negative effect of the treatment. The difference started to be observed since the second day after injection. The dissimilarity in survival between 0 μg 6-OHDA injected control flies and the experimentally doses injected flies was proportional to the increased doses of 6-OHDA. However some flies survived up to 7 days post treatment, even after being injected with 100μg of 6-OHDA.

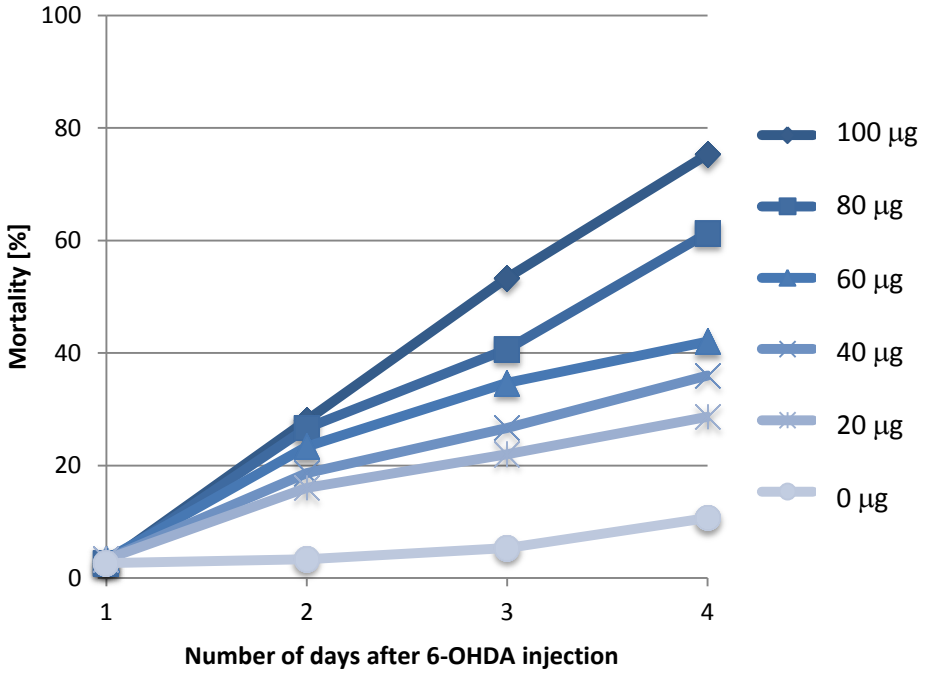


Fig. 5.7 Mortality curve illustrating the percentage of the cumulative mortality observed in sugar-fed flies injected with 100 µg, 80 µg, 60 µg, 40 µg, 20 µg and 0 µg 6-OHDA. The mortality increases with increasing dose of 6-OHDA (three biological replicates of 50 flies); mean.

Dose of 6OHDA [µg]	Cumulative mortality (n= 50 in each group)			
	Day 1	Day 2	Day 3	Day4
100	1.33 *± 0.277*	14.00 *± 0.480*	26.67 *± 0.555*	37.67 *± 0.734*
80	1.33 *± 0.277*	13.33 *± 0.277*	20.33 ± 0.734	30.67 *± 0.734*
60	1.67 *± 0.277*	11.67 *± 0.734*	17.33 ± 0.734	21.00 *± 0.480*
40	1.67 *± 0.277*	9.33 *± 0.555*	13.33 *± 0.277*	18.00 *± 0.480*
20	1.67 *± 0.277*	8.00 *± 0.832*	11.00 *± 0.480*	14.33 *± 0.277*
0	1.33 *± 0.277*	1.67 *± 0.277*	2.67 *± 0.277*	5.33 *± 0.277*

Table 5.1 The toxicity effect of different 100 µg, 80 µg, 60 µg, 40 µg, 20 µg and 0 µg doses 6OHDA. The mortality is increasing with the increasing dose of 6-OHDA (three biological replicates of 50 flies); mean *± SD*.

5.3.6 6-hydroxydopamine as an indirect regulator of ovarian development

For subsequent validation of the specific food-dependent reproductive strategy, flies were injected with different doses of 6-OHDA (as tested in the previous experiment) to down-regulate or prohibit their digestion. At that time, flies were reared on a liver diet over following days, after which a set of ten insects were sacrificed to determine their ovarian development. Measurement of the ovarian development (its diameter) took place between 9-11 AM, 1, 2 and 4 days after the treatment.

The results show a similar tendency as in the previous experiment; the maturation of eggs was suppressed according to the dose of 6-OHDA (Table 5.2).

Dose of 6OHDA [μg]	**Ovary size [mm] ±*SD***		
	Day 1	Day 2	Day 4
100	0.76 ± *0.037*	0.75 ± *0.041*	0.74 ± *0.042*
80	0.78 ± *0.042*	0.082 ± *0.062*	1.07 ± *0.078*
60	1.01 ± *0.088*	1.27 ± *0.134*	2.42 ± *0.120*
40	1.05 ± *0.083*	1.34 ± *0.063*	2.55 ± *0.076*
20	0.08 ± *0.059*	1.38 ± *0.042*	2.62 ± *0.059*
0	1.09 ± *0.046*	1.39 ± *0.053*	2.68 ± *0.086*

Table 5.2 Dose-dependent ovarian development observed in liver-fed flies injected with different doses of 6OHDA measured on 1, 2 and 4 days after the treatment. Increasing dose of 6-OHDA results in less developed ovaries; mean ± *SD*.

Flies that had constant access to liver and were injected with Ringer containing 0 μg 6-OHDA developed their eggs (Fig. 5.8). The changes in ovary size and YPs accumulation were clearly observed since the first day after liver feeding. The oogenesis process completed within 4 days and mature eggs of 8 days old females ovulated into the uterus ready to start embryogenesis.

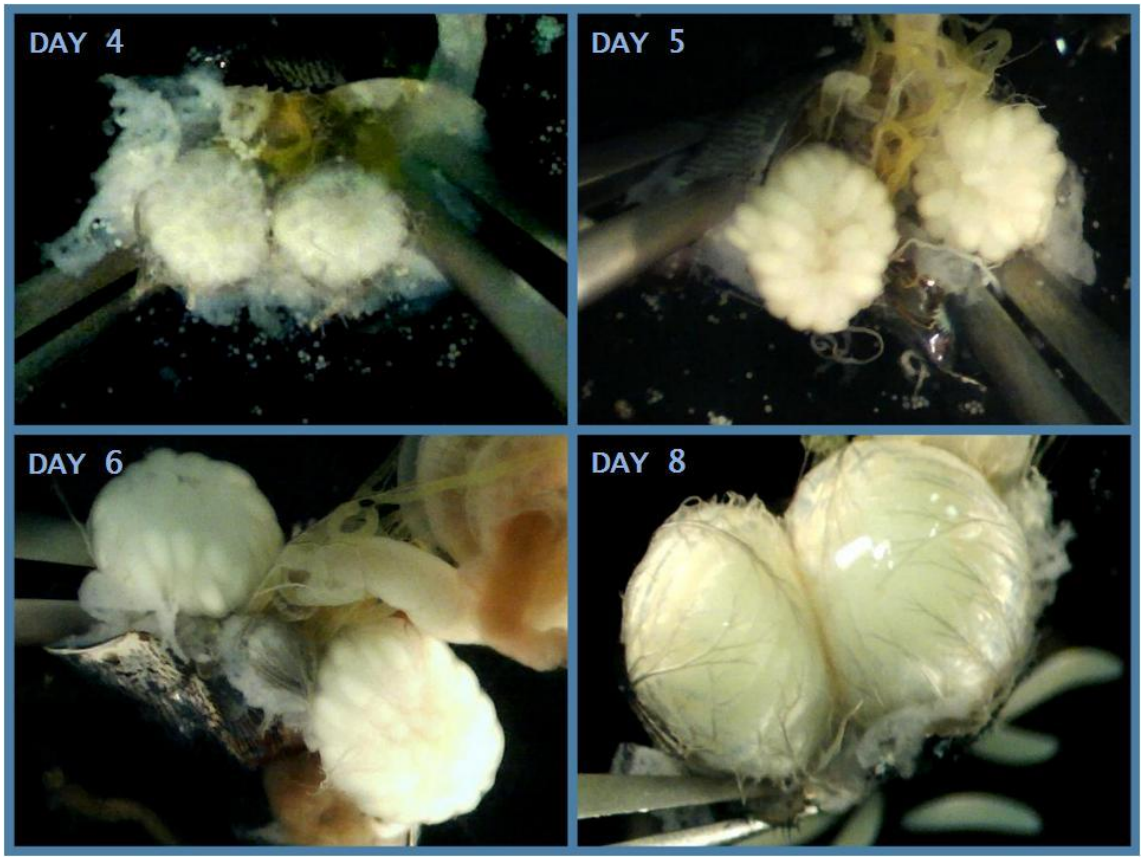


Fig. 5.8 Ovarian development in flies injected with Ringer (0 μg 6-OHDA), fed with liver at four days after emergence. Ovarian development of 4 days old sugar-fed flies (DAY 4), increased ovarian size observed after one and two days post liver feeding (DAY 5 and DAY 6 respectively), mature ovulated eggs translocated into the uterus bicornus after 4 days post liver feeding (DAY 8).

Two groups of insects treated with the smallest doses of 20 μg and 40 μg 6-OHDA also developed eggs normally; however, the ovaries of those flies were a little bit smaller in their size than those of the flies injected with Ringer only. Egg follicles of the flies injected with the three highest doses of 60 μg (0.3 M), 80 μg (0.4 M) and 100 μg (0.5 M) were much less developed and had not been transferred into the uterus. Compared to the starting point of ovaries of 4 days old sugar-fed flies, the ovaries of flies injected with the highest dose of 100 μg (0.5 M) 6-OHDA were smaller than at the beginning of the experiment (Fig. 5.9B).

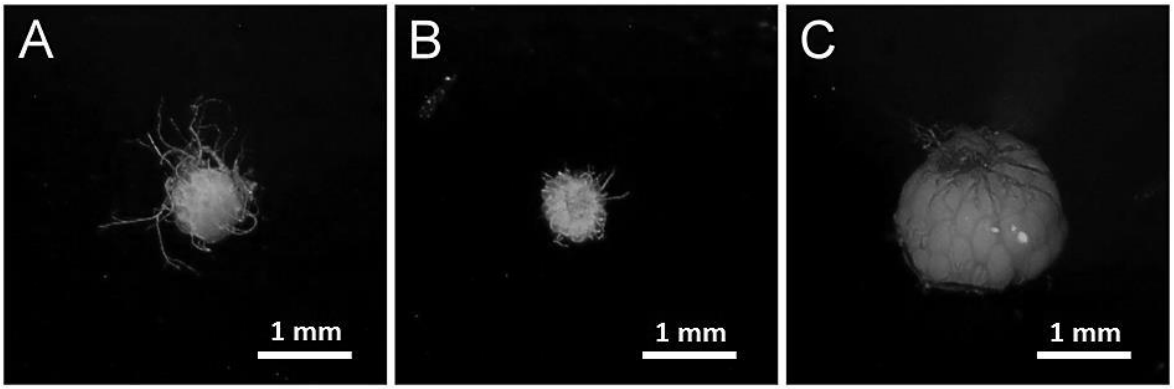


Fig. 5.9 Ovarian development under 6-OHDA treatment. Four days old insects were injected with Ringer's solution or 100μg 6-OHDA and fed with liver. No ovarian development was observed in oxidopamine treated flies. Ovary of 4 days old sugar-fed fly (A); ovary of liver-fed fly injected with 100 μg 6OHDA (2 days after the injection) (B); ovary of liver-fed fly injected with 1 μl Ringer's solution (2 days after the injection) (C).

5.3.7 Ovarian development under different nutritional condition

As demonstrated in the previous experiment, female flies fed with liver developed their eggs that were further translocated into the uterus for embryogenesis. Further experiments demonstrated that there was no observed difference in the ovarian development between flies that were fed with liver right after emergence or 4 days later (see time needed for potency). Additionally, there were no dissimilarities observed between insects that were continuously exposed to liver and a group that was offered the flesh meal for one hour only (data not shown).

As expected for anautogenous insects, female flies that were never exposed to any meat did not develop eggs. Their ovarian development was arrested at the stage observed at day 4 after emergence. Nevertheless there was some slight amount of YP aggregation observed in a few individual insects (Fig. 5.10).

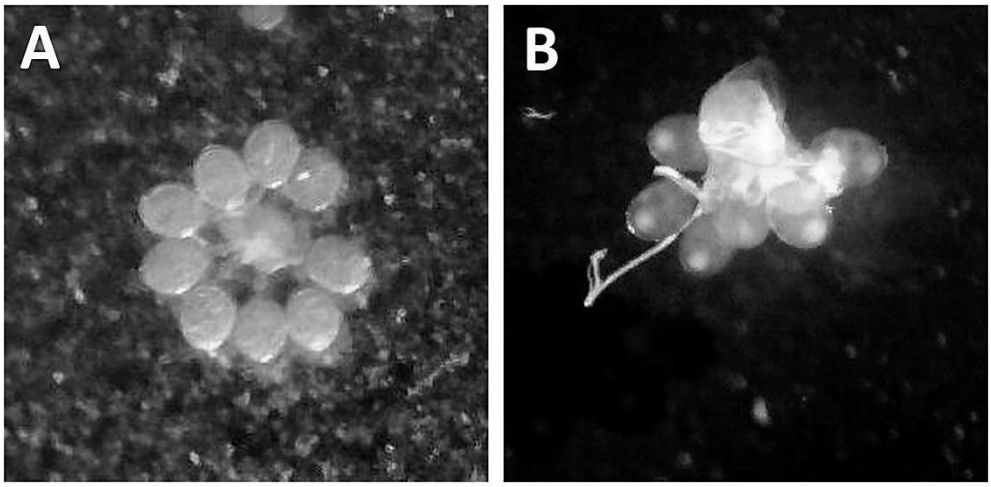


Fig. 5.10 Eggs development observed in sugar-fed flies never exposed to any protein meal. Empty oocytes that contain no YPs (A), and oocytes that accumulated some very small amount of YPs (B) observed in sugar-fed flies. No further egg development is observed beyond this previtellogenic stage.

The experimental feeding with an artificial diet containing pure protein (BSA) and salts solution, resulted in partial egg development. The size of the oocyte and YPs aggregation was two folds smaller, compared to proper physiological development (Fig. 5.11). 8 days old females did not translocate their eggs into the uterus. Even the prolonged time of the experiment did not show full ovarian development or ovulation. The yolk accumulation in artificial-fed flies consisted of approximately 50-75% of the eggs' volume.

The same, incomplete ovarian development was observed in the group of flies fed with free amino acids (data not shown).

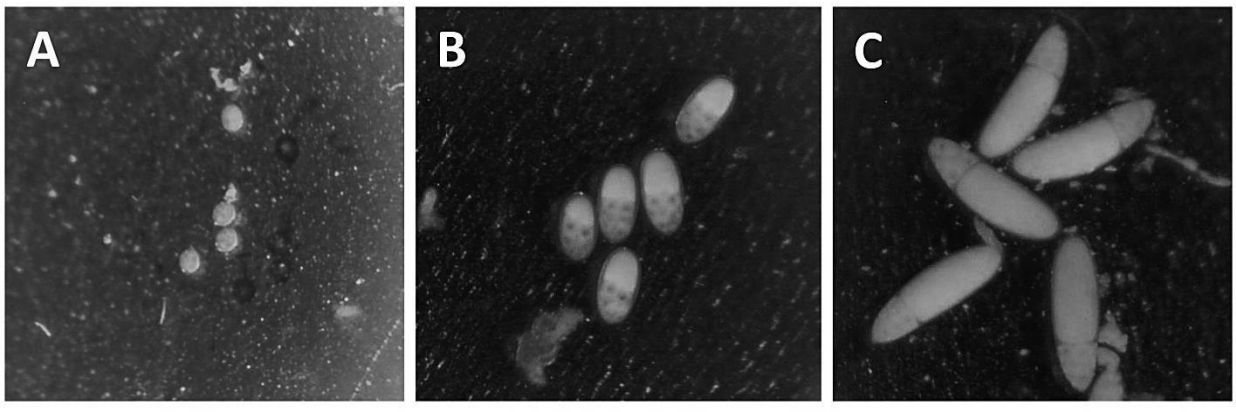


Fig. 5.11 Oocyte development of 6 days old flies under strict sugar and water diet (A), artificial protein diet (BSA) (B), and complete diet (liver) (C). No egg development is observed in non-protein-fed flies. Insects fed on artificial protein diet develope eggs much less than flies fed with liver as complete diet.

Protein meal digestion increases the level of free amino acids that circulate in insect hemolymph. In that experiment 4 days old sugar-fed flies were injected with free amino acids solution. That was done to mimic the increase of the free amino acid level that normally occurs post protein feeding and to bypass the feeding and digestion processes. Measurement of the ovarian development 1 day after injection (Fig. 5.12) and 3 days later, revealed that none of those flies was ale fully developed its ovaries. Similarly, as observed in the previous experiment, their size was two folds smaller than during normal physiological development and the eggs were still not ovulated. Also in those conditions, the extended observation time did not result in full development, or in translocation of the ovulated egg follicles into the uterus.

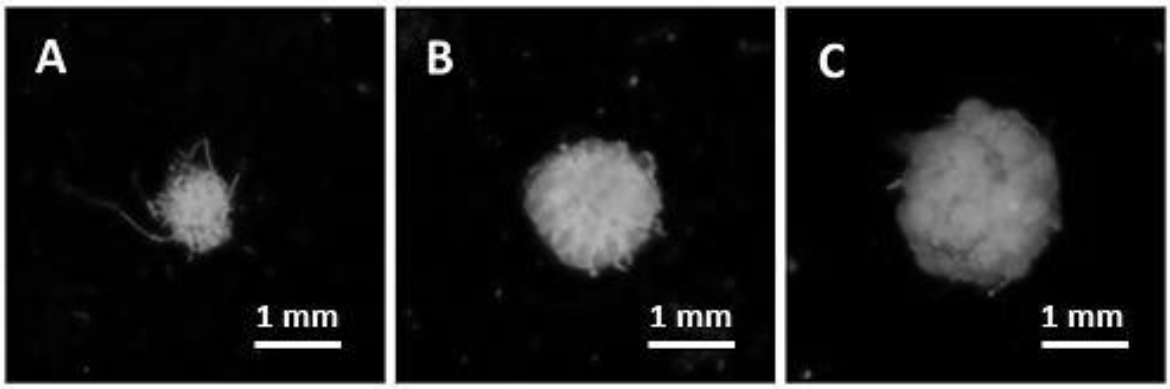


Fig. 5.12 Ovarian development of 4 days old sugar-fed flies flies (A), 5 days old sugar-fed injected with 1 μl of μM free amino acids solution (B) and 5 days old liver-fed flies (C). Increased amino acid concentration in the hemolymph results only in partial ovarian development.

A double injection of a free amino acid solution or even additional supplementation with 1 μg (2 mM) 20-hydroxyecdysone, a known stimulator of YP synthesis (Huybrechts & De Loof, 1981), did not stimulate full egg development (data not shown).

5.4 Discussion

Early post liver feeding decapitation results in prohibition of the normally observed increase of the proteolytic activity in the midgut. The same inhibition effect can be obtained by the injection of 6-OHDA into non-decapitated flies within 4 hours post liver feeding or before feeding. This chemical decapitation allows insects to keep their heads and mouthparts and

allow them to consume food. Nevertheless, the results confirm the previous findings that delayed post liver feeding mechanical or chemical decapitation (after 4 hours post liver feeding) no longer negatively influenced the ongoing digestion process. Based on the observed dose-response effect, we propose that using different doses of 6-OHDA can become a very handy tactic for experimental regulation of digestion (up to the particular wanted level) in anautogenous, as well as in autogenous insects. However, it is presently unknown if 6-OHDA blocks the signals directed to the brain or derived from the brain, as they are both shown to occur in insects (Mikani *et al.*, 2012). *In vivo* rescue experiments using dopamine, L-DOPA, epinephrine and norepinephrine in doses of 1ng-10μg did not succeed. Unfortunately we do not know the detailed mechanism of 6-OHDA action in insects; it is unknown if 6-OHDA destroyed only the neuronal connection within the brain. It is well possible that 6-OHDA additionally destroyed the CC as crucial neurohemal organ or alternatively interfered with any other organs/system needed for successful regulation of digestion in the studied anautogenous flesh fly model. Importantly, it seems unlikely that 6-OHDA directly interacted with the dopamine receptor, as earlier suggested (Nakamura & Thoenen, 1971). Indeed a competitive activation assay for D2-like dopamine receptor of *Tribolium castaneum* (Verlinden *et al.*, 2015) expressed in mammalian cells, showed no binding affinity of 6-OHDA to the receptor, up to a concentration of 10^{-6} M (data not shown).

Nevertheless, the results of the enzymatic activity assay clearly show the ability of 6-OHDA to prevent an increase in midgut proteolytic activity imposed by liver feeding, and this is in spite of the presence of proteins and non-proteinaceous food components in the midgut. This observation suggests the inhibition of the proteolytic enzyme expression. Further examination of trypsin gene transcript levels resulted in an increase observed in both liver-fed and in 6-OHDA injected liver-fed flies. This evidence indicates that 6-OHDA inhibits trypsin gene expression on a posttranscriptional level. Remarkably, the ovary derived TMOF of *Neobellieria bullata* also inhibits late trypsin translation (Bylemans *et al.*, 1994;Borovsky *et al.*, 1996).

Huybrechts and De Loof (1981) in agreement with an earlier report on 6-OHDA in the cockroach, *Periplaneta americana,* (Hentschel, 1975;Hentschel, 1980) also demonstrated the 6-OHDA inhibitory effect on egg development in the anautogenous flesh fly *Neobellieria*

bullata. Our study of the effect of 6-OHDA at the level of midgut proteolytic activity and the herein described experiments strongly suggest that the inhibition of vitellogenesis in *Sarcophaga* is a secondary effect, because 6-OHDA most likely acts directly on the midgut by inhibiting dopaminergic neural signaling. Without such dopaminergic signaling (either anterograde or retrograde), the posttranslational activation of trypsin does not occur. The observed requirement of dopaminergic signaling in the regulation of protein meal induced egg development is in full agreement with the observation in anautogenous *Aedes aegypti,* in which a drastic increase of dopamine levels occur in the head of post blood meal females (Andersen *et al.*, 2006).

In *Sarcophaga crassipalpis,* the digestion process is most probably not only crucial for reproduction but also for the organism in general to maintain a proper physiological state. Indeed, inhibition of the midgut activity by 6-OHDA seemingly results in reduced longevity in a dose-dependent way. Although a direct overall toxic effect is rather not apparent within 24 hours, a long lasting, slow acting overall toxic effect of this neurotoxin cannot be excluded. And although mortality due to 6-OHDA injections in insects has not been reported before, the general toxicity/mortality effects should be taken into account. While planning experiments using pharmacological doses of 6-OHDA, it is suggested that a large starting number of insects should be used, especially for long duration experiments.

In addition to the above considerations about the mode of action of 6-OHDA in the fly, we refer to the recent finding that 6-OHDA prevents miRNA loading to protein argonaute-2 (Ago2) and hitherto, the formation of a Risk silencing complex *in vitro,* suggesting the possibility that 6-OHDA inhibits RNAi regulated gene expression. Although this RNAi inhibition effect of 6-OHDA is not confirmed using human kidney derived T-Rex 293 cells (Tan *et al.*, 2012) and we neither did observe any clear RNAi inhibiting effect of 6-OHDA (50µM) in *Drosophila* S-2 cells (unpublished results), this report should be taken into consideration as it cannot yet be excluded that following uptake into dopaminergic neurons, 6-OHDA exerts its effect with an instant release of a selective RNAi based block. Possibly, 6-OHDA enters the midgut cells and exerts its instant RNAi inhibiting effect at the level of translational protease activation (Fig. 5.13). It is likely that some translational factor inhibits trypsin (proteases) gene expression, keeping it at relatively low level, up to the moment of the protein meal intake. A food-derived signal might activate micro RNA synthesis, which

results in RNA silencing and post-transcriptional down-regulation of the trypsin inhibitor, and it consequently increases proteolytic activity post liver feeding. 6-OHDA injection is reported to prevent formation of the miRISC complex (Tan *et al.*, 2012) and if this is also happening *in vivo,* the translation of the trypsin inhibitory factor will be not stopped. This would then result in very low proteolytic activity, as observed in decapitated liver-fed flies.

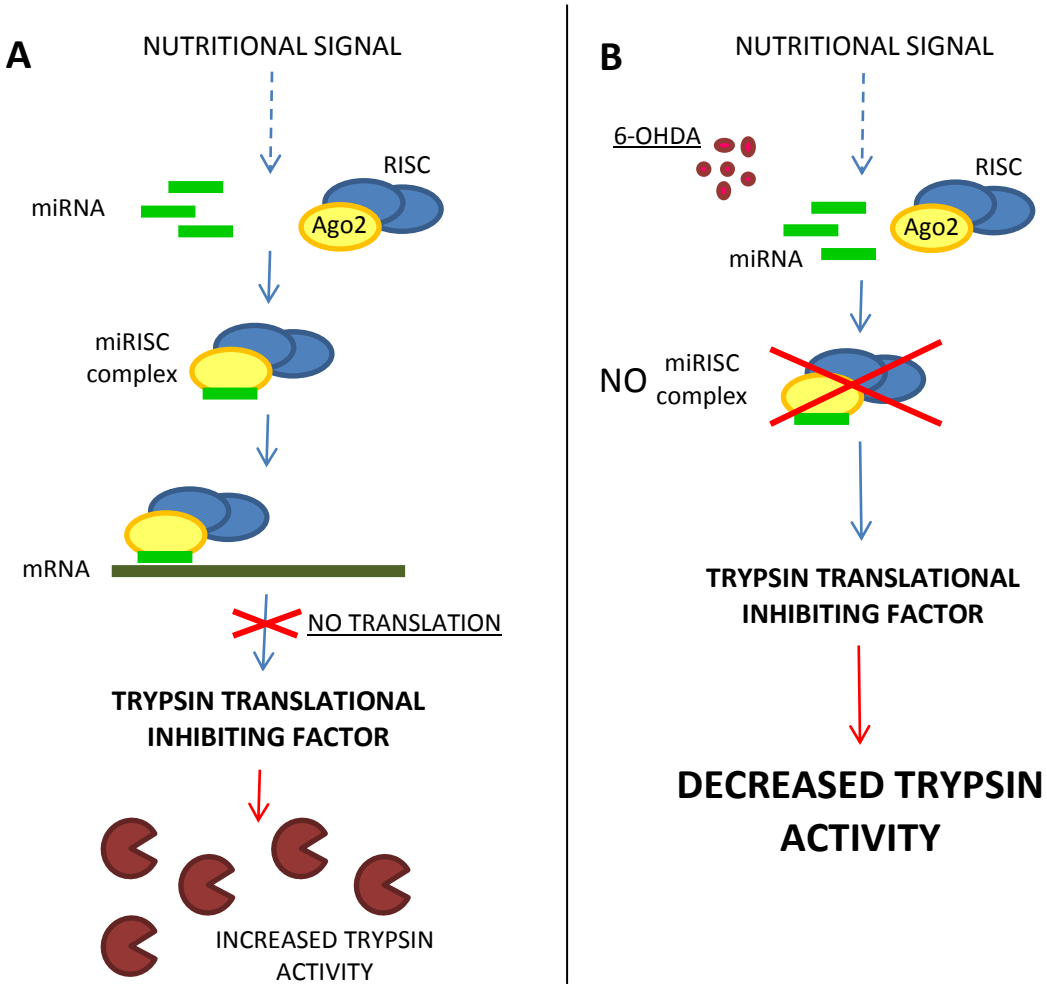


Fig.5.13 Hypothetical model, which can explain the arrested midgut proteolytic activity in liver-fed flies, under 6-OHDA treatment. It is well possible that trypsin gene expression is post-transcriptionally inhibited by an additional translational factor. This inhibiting factor will be silenced via a miRNA mechanism at the moment of the protein meal intake, which will result in higher midgut proteolytic activity (A). Injected 6-OHDA might prohibit the formation of the miRISC complex and, as a result, the trypsin translational inhibiting factor is continuously expressed, and hitherto very low proteolytic activity is observed (B).

The latter would explain our experimental failure in rescuing the 6-OHDA induced effect by directly targeting the dopamine or adrenaline postsynaptic receptors. This way the assumed

neurodegeneration caused by 6-OHDA, which takes time to be established, in our model would only explain the long-term toxicity and might not be directly responsible for the prevention of protease activation as such.

In anautogenous insects, only appropriate feeding, digestion and absorption of a piece of real meat containing proteins, lipids and carbohydrates as complete diet, is able to assure the adequate nutrition required for complete ovarian development.

The experimental results highly suggest that an increased quantity of free amino acids, circulating in the hemolymph after either feeding with artificial diet or following intra-abdominal injection is a required factor to start vitellogenesis but not sufficient for full egg maturation. The essential role amino acids are playing in ovarian development initiation was also observed in several anautogenous mosquito species (Uchida *et al.*, 2001;Uchida *et al.*, 2003). As observed in the flesh fly, not all amino acid fed/injected mosquito species were able to complete oogenesis, which suggests the requirement of some other essential (regulatory) factors.

Elimination of the digestion process by feeding on a strict amino acid diet resulted in failure of ovarian development in the mosquito, *Culex pipiens* (Uchida & Katsuhiko, 1982). This strongly supports our observations of amino acids meal being not able to stimulate any increase in midgut digestive activity (chapter 2), and consequently the observed lack of completed oogenesis. It is suggested that there is some particular compound(s) present in the midgut endocrine cells that is released into the hemolymph during the intensive digestion process or during nutrient absorption (Clements, 1992;Briegel *et al.*, 2003).

The fact that 20-hydroxyecdysone plays a crucial role in large scale YP biosynthesis, is very well known. This steroid hormone seems to be a very powerful tool, due to the fact that it is even able to start vitellogenesis in male insects (Huybrechts & De Loof, 1981). Nonetheless, this ecdysteroid alone does not suffice to stimulate full maturate ovaries, even when used in combination with injected free amino acids. The same limited oocyte development under similar treatments was observed in mosquitoes (Spielman *et al.*, 1971). The arrested egg development state is maintained until proper protein feeding.

Incomplete ovarian development can be also caused by a lack of lipids in the artificial experimental diet, as lipids are one of the principal components of the ovarian yolk (Clements, 1992;Sawabe & Moribayashi, 2000). In favor of this latter hypothesis are the studies that presented linoleic and arachidonic acids as 1) being involved in vitellogenesis, 2) being a releaser of reproductive hormones or 3) acting directly on these reproductive hormones (Guilvard *et al.*, 1984). Unfortunately all our attempts to supplement pure lipids into fly artificial diets failed. During the feeding process, flies spread the lipids all over their body surface causing a permanent closure of the spiracles that normally allow air to get into the respiratory system. This always resulted almost instantly in fast death of the experimental animals.

CHAPTER 6.

General conclusions and future perspectives

The main aim of this research work was to gain a better understanding of anautogenous reproduction. This is because insects with this kind of reproductive strategy have a significant impact on human health (e.g. disease spreading mosquitoes), as well as on animal farming (e.g. myiasis causing flesh flies). Therefore, using a combination of enzymatic assays, decapitation and rescue experiments, peptidomics and molecular biology approaches; we investigated the anautogenous reproductive condition and its possible control mechanisms in the anautogenous flesh fly, *Sarcophaga crassiapalpis*.

Anautogeny, defined as the need of an adult female to consume an additional proteinaceous meal to start oogenesis (Attardo *et al.*, 2005) implies that this phenomenon consists of three actions which depend on each other: the protein meal intake, its subsequent digestion and vitellogenesis.

Regarding the first two aspects (protein meal intake and its digestion) of this food-dependent reproduction, our anautogenous flesh fly, *S. crassipalpis*, seems to be a good model organism to study this specific egg development strategy. Similar to mosquitoes, only female flies fed with the proper complex meal of liver (blood in mosquitoes) are able to fully mature and deliver their eggs (Uchida & Katsuhiko, 1982;Arsic & Guerin, 2008). Another common feature observed in both of these anautogenous insects is the increase of the midgut digestive activity elicited by the protein food intake (Okuda *et al.*, 2005).

Nevertheless, the third aspect (vitellogenesis) of the food-induced vitellogenesis seems to be different in flies and mosquitoes. They both have an ecdysone hormone that is hydroxylated to the 20-hydrohyecdysone, which then triggers large scale yolk protein production by the fat body and follicle cells (Huybrechts & De Loof, 1977;Briers & De Loof, 1980;Huybrechts & De Loof, 1981;Huybrechts, 1982;Briers & Huybrechts, 1984; Raikhel *et*

al., 2002). In mosquitoes, the blood meal also stimulates the secretion of a peptide hormone called ovarian ecdysiotropic hormone (OEH) (Brown *et al.*, 1998), whereas in flesh flies, the OEH orthologue seems to be missing. Genome blasting highlights the existence of OEH orthologous genes in all mosquito species but the exact mode of action of this hormone remains unknown (Badisco *et al.*, 2007;Dhara *et al.*, 2013;Vogel *et al.*, 2015). Motif-based blast using the mosquito consensus sequence does not allow confirmation of whether there is an occurrence of the homologous gonadotrophin in anautogenous flies. Some former studies indicated the isolation of an ecdysteroidogenic hormone from the house fly, *Musca domestica*, a likely homologue of the mosquito OEH. It was reported to stimulate ovarian ecdysone production *in vivo* and *in vitro* not only in the fly, *Musca domestica*, but also in the mosquito, *Aedes aegypti* (Masler & Adams, 1986;Adams *et al.*, 1997). Surprisingly, this hormone was present in both female and male flies but its function in males was unknown. Apart from these two publications that report the isolation of the compound demonstrating OEH activity, having a molecular mass of 8.1 kDa (similar to OEH isolated and identified from *Ae. aegypti*), the research for identification of such a compound was seemingly discontinued as no further publications on this subject (from this or any other research group) can be traced in the literature. Additionally, a sequence similar to mosquito OEH, cannot be found in the housefly genome.

This all implies that there are some significant dissimilarities between anautogenous flies and anautogenous mosquitoes. Nevertheless, both of them share (so far) the same digestive nature which allows for studying this initial part of the anautogenous reproductive strategy, using *S. crassipalpis* as a model organism.

The majority of the proteolytic activity in the entire order of Diptera is represented by the serine protease trypsin (Borovsky *et al.*, 1996), which, as mentioned before, exists in anautogenous insects in two food-dependent forms (early and late trypsin). Previously, all authors suggested that early trypsin was present and active in only the first phase of the meal digestion and it was later entirely replaced by its ‘late’ form. Nowadays it is known that the proteolytic digestion process is much more complex and both enzymes work together in the later phase of proteolysis (Brackney *et al.*, 2008;Dias-Lopes *et al.*, 2015).

Interestingly, it is very unlikely to find the name 'chymotrypsin' in the literature concerning digestion in anautogenous insects; whilst this term is in common use in the publications referring to digestive activity in other insects (Spit *et al.*, 2014).

Nonetheless, the detailed analysis of *Neobelliera bullata* and *Aedes aegypti* amino acid sequences which encode early and late trypsin, revealed that late trypsin protein contains several structural features, such as some residues that determine the substrate specificity pocket, which is characteristic for the chymotrypsin superfamily (Borovsky *et al.*, 1996;Jiang *et al.*, 1997). More recently, large-scale phylogenetic analysis of insect trypsin and chymotrypsin amino acid sequences have indicated that all dipteran late trypsin sequences cluster together within the chymotrypsin group (Marshall *et al.*, 2008;Spit *et al.*, 2014). Spit et al. (2014) even point to the lack of amino acid motifs that are characteristic for trypsin-like enzymes in these late trypsin sequences.

Our results confirm that trypsin is the main proteolytic contributor and it was observed in both sugar and liver-fed flies. A different situation was observed for chymotrypsin, which demonstrated a very low activity in sugar-fed flies and had significant participation in protein digestion of liver-fed flies (as is the case for late trypsin). Altogether, the results of the amino acid sequence analysis, food-dependent gene expression and the use of enzyme-specific substrate or inhibitor assays, strongly indicated that dipteran late trypsin is true chymotrypsin.

There is a great amount of evidence in the literature about the neuropeptidergic regulation of feeding/digestion in insects. Neuropeptidergic digestion control is initiated through external and internal stimuli and mainly influences gut motility and enzymatic activity (reviewed by Audsley & Weaver, 2009).

Studies from last years, provide more and more evidence to indicate that the adipokinetic hormone is involved in the stimulation of digestive enzymes. This is very well documented in the firebug, *Pyrrhocoris apterus*, where AKH treatment significantly increases protein digestion in the midgut (Kodrik *et al.*, 2012); however the effect is only observed *in vivo*. This author suggests that AKH may influence feeding behavior, since the AKH-treated insects consume more food and as such, the increased digestive activity might be stimulated

secondary, by stretch receptors that monitor a filling of the midgut; otherwise, this may be controlled by other mechanisms. Similar results were also observed in the salivary glands of this firebug (Vinokurov *et al.*, 2014). This author additionally pointed to the fact that AKH-increased enzymatic activity is observed some hours after the treatment as no effect is observed immediately after the hormone injection. This suggests that there is an involvement of a more complicated pathway or cascade in this digestion stimulatory mechanism. The first (and so far one and only) direct effect of the AKH treatment on the midgut digestive activity was observed in the cockroach, *Periplaneta americana* (Bodláková *et al.*, 2016), where an increased digestive activity was observed both *in vivo* and *in vitro*.

In the flesh fly, *S. crassipalpis*, AKH injection stimulated midgut proteolytic activity in both sugar and liver-fed flies. However, this stimulatory effect was observed in *in vivo* conditions only. The theory suggested by Kodrik et al. (2012) regarding AKH influence feeding behavior (AKH-treated insects consume more food) and consequent increased digestion due to the full midgut, seems to be not the case in our insect model; since decapitated liver-fed flies (full midgut) did not demonstrate any increase in midgut proteolytic activity. According to the remark of the second researcher, Vinokurov et al. (2014), the AKH stimulatory action is not observed directly after the hormone treatment (the maximal enzymatic activity of decapitated liver-fed flies is observed some hours later). Altogether, the AKH-dependent digestion stimulation mode of action remains unknown and seems to be rather indirect.

Interestingly, there were the differences in the adipokinetic hormone receptor gene expression that were observed between sugar and liver-fed flies. The post protein feeding decreased AKHR transcript levels suggests that this receptor works as a kind of energy safety valve. A high AKHR gene expression in sugar-fed flies should allow for mobilization of the stored energy to assure all basic physiological processes; whilst its reduced presence in the liver-fed flies, protected their stored energy supplies (to save it for a later time or emergency) and forced 'new' food-derived energy to be used beforehand. Similar decreased AKHR transcript levels were also observed in the blood-fed mosquito, *Anopheles gambiae* (Kaufmann & Brown, 2006), which is also an anautogenous insect. If both these anautogenous insects share the AKHR food-dependent expression nature, it is also possible that they can share similar AKH stimulatory effects on their digestive enzymes.

According to our findings, the rarely used neurotoxin, 6-hydroxydopamine, might become a potential research tool in insect study. Its capability to evoke chemical decapitation under continued feeding conditions, allowed us to study the regulatory effects of different physiological processes in autogenous and anautogenous insects. Nevertheless, much more research is required to better understand the detailed mode of action of this neurotoxin in insects and to establish if it only damages neurons or also causes deleterious effects on other cells. To validate whether 6-OHDA could be used as a common research tool in insect physiology, an entire investigation of all changes resulting from chemical decapitation is needed, since the toxic damage to different cells or even organs cannot yet be excluded. The results of our experiments indicate that flesh flies are able to withstand this harsh treatment for several days. However, it would be interesting to examine if a post liver feeding injection (after the critical moment of 4 hours post protein feeding) would allow them to fully develop ovaries and deliver offspring; also if they would be able to generate and mature the second batch. In order to have a wider view about the influence of this neurotoxin on each developmental stage, it would also be worth testing the influence of 6-OHDA on fly maggots (will they develop and undergo metamorphosis or not?).

All results of this study contributed to a better understanding of the digestion process, which is the first aspect of the anautogenous reproductive strategy under study. Nonetheless, much more research is still required to fully understand all parts of this specific reproductive condition.

Better investigation of the *S. crassipalpis* gut and the stomatogastric nervous system, including neuronal connections and all possible (neuro-)peptides and their receptors would for sure provide some additional knowledge about the feeding/digestion process. This would fully allow us to understand which factors and which regulatory mechanisms are responsible for the increase in proteolytic activity, as observed in the midguts of liver-fed flies. Do anautogenous flies share the same digestion regulatory mechanisms as anautogenous mosquitoes?

The matter of signal/signaling pathways that inform the brain of the anautogenous insects about the protein meal intake and sufficient energy levels to start vitellogenesis remains unresolved. Our results are consistent with the observations in the mosquito, *Culex pipiens*,

that this signal/signaling pathway is activated during protein digestion, since the elimination of this process results in the failure of ovarian development (Uchida & Katsuhiko, 1982). Therefore, better understanding of midgut characteristics could directly answer this question or create some potential directions in further research.

Admittedly, the relatively new studies that indicate that the adipokinetic hormone plays a role in digestion regulation, definitely need further examination; since this theory still leaves a lot of questions unanswered. The most important, but also the most complicated is the investigation of the pathway(s) that results in AKH-mediated enzymatic activity stimulation. The difficulty to resolve this issue lies in the fact that any combination of the *in vitro* isolated midgut and any other tissue does not raise digestive activity, whilst a strong stimulatory response by AKH was observed in *in vivo*. Attention should be also paid to the selectivity of the enzymes that are stimulated by this hormone: AKH does not increase the activity of all digestive enzymes, but only particular ones like peptidase and glucosidase in the midgut of the firebug (Kodrik *et al*., 2012) and lipase, glucosidase and amylase in the salivary glands of this bug (Vinokurow *et al.*, 2014). It would be interesting to verify if this selective stimulation is also valid in the flesh fly model. Regarding the common anautogeny feature and similar food-dependent AKHR gene expression, it would be also worth to test if AKH demonstrates any stimulatory effect on blood digestion in mosquitoes.

Clearly, still not everything is known about the phenomenon of anautogenicity in any insect. Nevertheless, our study provides some contribution concerning the control of digestion that definitely should be verified in the anautogenous mosquito model organism.

The comparison of both the flesh fly and mosquito anautogenous models, builds upon previous knowledge and newly obtained information, and also pinpoints the yet unknown regulatory aspects is graphically presented below (Fig. 6.1).

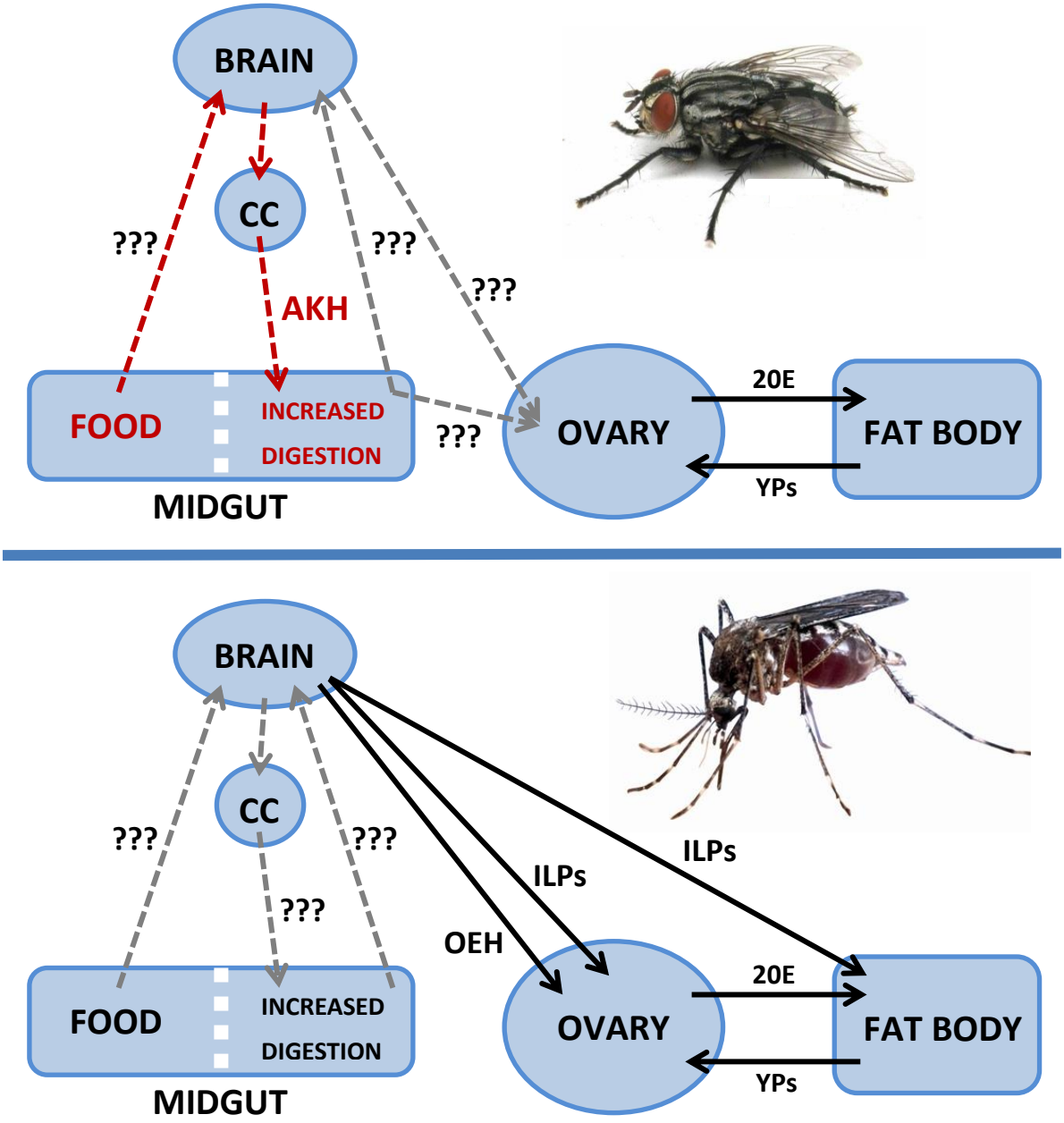


Fig. 6.1 The comparison of the anautogenicity models in flies and mosquitoes. In the anautogenous flesh fly, food-derived signal stimulates brain to release AKH stored in the CC. It results in increased digestive activity which stimulates brain or directly stimulates ovaries for ecdysone production. Hydroxylated ecdysone enforces fat body for a large scale yolk production and its uptake by developing ovaries (top schema). In the anautogenous mosquito, food-derived signal also signals to the brain which results in increased digestive activity; however the influence of the AKH was never tested in these insects. Some additional signal stimulates brain for OEH and ILPs release and stimulation of the ovaries for ecdysteroidogenesis which results in yolk protein production by the fat body and ovarian development (bottom schema). Red color indicates new knowledge obtained from this research, gray color indicates unknown pathways, black color indicates already known facts; whilst dashed arrows symbolize unknown mechanism. Abbreviations: 20E, 20-hydroxyecdysone; AKH, adipokinetic hormone; CC, *corpora cardiaca*; ILPs, insulin-like peptides; YPs, yolk proteins. Image credits: insects-net.fr and 40.media.tumblr com respectively

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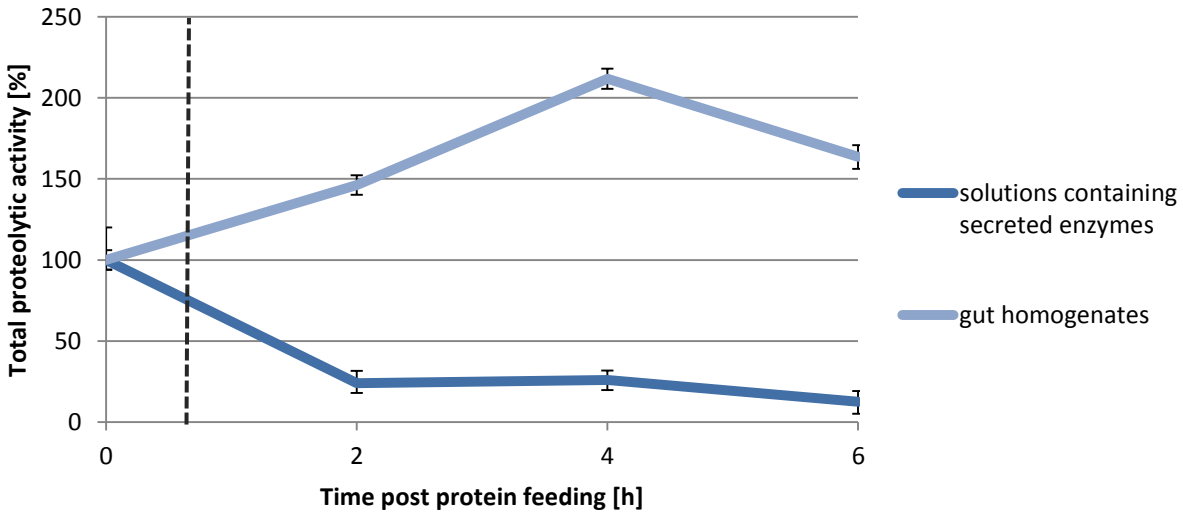
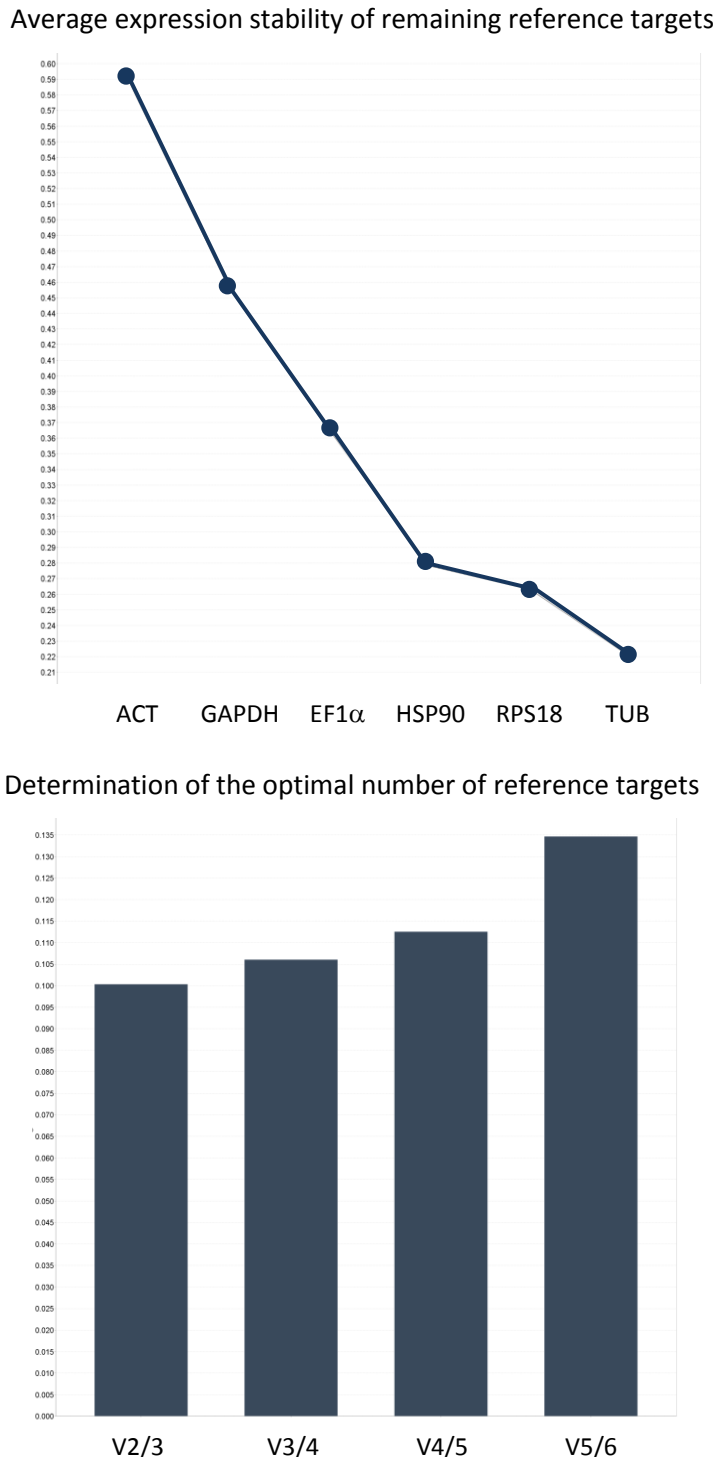
Supplementary data



Supp. fig. 2.1 The comparison of the results of midgut proteolytic activity demonstrated by gut homogenates and solutions containing secreted enzymes. Samples of both conditions were incubated with the same amount of azocasein substrate, and for the same amount of time. The homogenated samples show clear increase in the midgut enzymatic activity induced by protein feeding, in contrast to the solution samples containing secreted enzymes, that present decreasing digestive activity profile. Dashed line indicates the end of feeding time (4 replicates of 3 pooled midguts each); mean *± SD.*



Supp. fig. 2.2 The geNorm analysis of the housekeeping genes used for qRT-PCR measurement. The average expression stability of all reference genes starting with the least stable genes on the left side and ending with the most stable genes on the right side (top chart). The levels of variations in average reference genes stability with the sequential addition of each housekeeping gene for calculation of the normalization factor of the experiment (bottom chart). Based on the results of these analyses, the housekeeping genes selected for this experiment are: TUB, RPS18 and HSP90. Abbreviations: EF1α, elongation factor 1α; HSP90, heat shock protein 90; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPS18, ribosomal protein S18; TUB, tubulin; ACT, actin.

EARLY TRYPSIN

```
Aedae      ---------MNQFLFVSFCALLGLSQ------------VSAATLSSGRIVGGFQIDIAEV
Anoga      MISNKIAILLAVLVVAVACAQARVALKHRSVQALPRFLPRPQYDVGHRIVGGFEIDVSET
Culqu      ---------MKLFLLLATCAVAATAYPGTPQR--PRPWWNSLEATEGRIVGGFEVDIKDV
                    :  ::.   **    :                      ******::*: :.

Aedae      PHQVSLQRSGRHFCGGSIISPRWVLTAAHCTTNTDPAAYTIRAGSTDRTNGGIIVKVKSV
Anoga      PYQVSLQYFNSHRCGGSVLNSKWILTAAHCTVNLQPSSLAVRLGSSRHASGGTVVRVARV
Culqu      PYQVSLRSFGSHICGGSIISKRWILTAAHCASSADRPKETIRVGSSEKGSGGQILKLKRI
           *:****:    * ****::. :*:******: . :    ::* **: : .** ::::  :

Aedae      IPHPQYNGDTYNYDFSLLELDESIGFSRSIEAIALPEASETVADGAMCTVSGWGDTKNVF
Anoga      LEHPNYDDSTIDYDFSLMELETELTFSDVVQPVSLPEQDEAVEDGTMTTVSGWGNTQSAA
Culqu      VQHPQYDGSIIDYDFSLLELAEELELDDSHTTIALPEQDEPVTDGAICRVSGWGNTQSSA
           : **:*: .  :*****:**  .: :.     ::*** .* * **::  *****:*:.

Aedae      EMNTLLRAVNVPSYNQAECAAALVNVVPVTEQMICAGYAAGGKDSCQGDSGGPLVFGDEL
Anoga      ESNAILRAANIPTVNQKECTIAYSSSGGITDRMLCAGYKRGGKDACQGDSGGPLVVDGKL
Culqu      QSNKFLRATDVPSVNQDKCSEAYSDFGGVTPRMICAGYQEGGKDACQGDSGGPLVSGGKL
           : * :***.::*: ** :*: *  .   :* :*:****  ****:**********   :*

Aedae      VGVVSWGKGCALPNLPGVYARVSTVRQWIREVSEV
Anoga      VGVVSWGFGCAMPGYPGVYARVAVVRNWVRENSGA
Culqu      VGVVSWGYGCAVAGYPDVYSQIASVRDWIKEVSDV
           ******* ***:   * **:::: **:*::* * .
```

LATE TRYPSIN

```
Aedae      --MFTS---TVVFASLMA-----LASAF----PSLDNGRVVNGQTATLGQFPFQVLLKVE
Ochep      --MFAS---TVIIASFVA-----LSAAY----PSIDQGRVVNGQTATLGQFPYQVLLRIQ
Culqu      MNQFITLLATVACVALVQGASTARHPARPWWNAPPSSERIVGGFEIDILEVPYQI----S
              * :   **  .:::         *        .. *:* *    : :.*:*:    .

Aedae      LSQGRALCGGSLLSDQWVLTAGHCTDGAKS-FEVTLGAVDFEDTTNDGRVVLTATEYHRH
Ochep      FAEGKALCGGSLLSNQWVLTAGHCTDGAKS-FEVTLGAVDFNSETDDGRVVLTATEYYRH
Culqu      LQSYGHFCGGSIIGENWVLTAGHCADDNDVGLNVRVGS----SLHGSGGQLVPVKPVIQH
           : .   :****::.::********:*  .  ::* :*:    .   .*  :: ..   :*

Aedae      EKYNPLFATNDVAVVKLPTPVAFNDRVQPVKLPTGSDTFTD-REVVVSGWGLQKNGGNVA
Ochep      EKYNPLFATNDVAVVKLPQPVEFNDRVHPVELPSGPDSYAN-QEVVVSGWGLQKNGGNVA
Culqu      PQYNPSTIDFDFALLELEQPVQLSEEFFPVELPEQDQEVEDGQLLQVSGWGYTQNPSESN
            :***     *.*:::*  ** :.:.. **:**   :   : : : *****  :* .:

Aedae      DKLQYAPLTVISNNECSKAYSPLVIKKTTLCAKG-ENKESPCQGDSGGPLVLEGENVQVG
Ochep      DKLQYAPLTVITNDECSQTYSPLVIKKTTLCAKG-GNKESPCNGDSGGPLVLEGSKVQVG
Culqu      EALRATNVPAVSQEECRESYGGYQITDRMICAGYQAGGKDACQGDSGGPLVEG--KTLVG
           : *: : : .::::** ::*.   *..  :**      :. *:********    :. **

Aedae      VVSFGHAVGCEQGYPGAFARLTSFVDWIKQKTGL
Ochep      VVSFGHAAGCELGYPGAFARVTSFVDWVKKKTGL
Culqu      VVSWGIG-CAEPGYPGVYSREAAVRDWIKEHSGI
           ***:* .  .* ****.::* ::. **:*:::*:
```

Supp. fig. 2.3 Amino acid sequence alignments of early and late trypsins. Identical amino acid residues between sequences are markted by asterisk, whereas conserved residues are marked by colon. Common amino acid motifs for both trypsin forms are colored in black, whereas early or late trypsin specific motifs are colored in gray. Abbreviations: Aedae, *Aedes aegypti*; Anoga, *Anopheles gambae*; Culqu, *Culex quinquefasciatus*; Ochep, *Ochlerotatus epactius*.

EARLY TRYPSIN AMINO ACID SEQUENCES

>Aedes egypti (GenBank Acc. No. **AAM34268.1**)

MNQFLFVSFCALLGLSQVSAATLSSGRIVGGFQIDIAEVPHQVSLQRSGRHFCGGSIISPRWVLTAAHCTTNTDPAA
YTIRAGSTDRTNGGIIVKVKSVIPHPQYNGDTYNYDFSLLELDESIGFSRSIEAIALPEASETVADGAMCTVSGWGDTK
NVFEMNTLLRAVNVPSYNQAECAAALVNVVPVTEQMICAGYAAGGKDSCQGDSGGPLVFGDELVGVVSWGKGC
ALPNLPGVYARVSTVRQWIREVSEV

>Anopheles gambiae (GenBank Acc. No. **CAA80517.1**)

MISNKIAILLAVLVVAVACAQARVALKHRSVQALPRFLPRPQYDVGHRIVGGFEIDVSETPYQVSLQYFNSHRCGGS
VLNSKWILTAAHCTVNLQPSSLAVRLGSSRHASGGTVVRVARVLEHPNYDDSTIDYDFSLMELETELTFSDVVQPVS
LPEQDEAVEDGTMTTVSGWGNTQSAAESNAILRAANIPTVNQKECTIAYSSSGGITDRMLCAGYKRGGKDACQGD
SGGPLVVDGKLVGVVSWGFGCAMPGYPGVYARVAVVRNWVRENSGA

>Culex quinquefasciatus (GenBank Acc. No. **AAK50138.1**)

MKLFLLLATCAVAATAYPGTPQRPRPWWNSLEATEGRIVGGFEVDIKDVPYQVSLRSFGSHICGGSIISKRWILTAA
HCASSADRPKETIRVGSSEKGSGGQILKLKRIVQHPQYDGSIIDYDFSLLELAEELELDDSHTTIALPEQDEPVTDGAIC
RVSGWGNTQSSAQSNKFLRATDVPSVNQDKCSEAYSDFGGVTPRMICAGYQEGGKDACQGDSGGPLVSGGKLV
GVVSWGYGCAVAGYPDVYSQIASVRDWIKEVSDV

LATE TRYPSIN AMINO ACID SEQUENCES

>Aedes aegypti (GenBank Acc. No. **AAF82286.1**)

MFTSTVVFASLMALASAFPSLDNGRVVNGQTATLGQFPFQVLLKVELSQGRALCGGSLLSDQWVLTAGHCTDGAK
SFEVTLGAVDFEDTTNDGRVVLTATEYHRHEKYNPLFATNDVAVVKLPTPVAFNDRVQPVKLPTGSDTFTDREVVV
SGWGLQKNGGNVADKLQYAPLTVISNNECSKAYSPLVIKKTTLCAKGENKESPCQGDSGGPLVLEGENVQVGVVSF
GHAVGCEQGYPGAFARLTSFVDWIKQKTGL

> Culex quinquefasciatus (GenBank Acc. No. **AAB37261.1**)

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GHCADDNDVGLNVRVGSSLHGSGGQLVPVKPVIQHPQYNPSTIDFDFALLELEQPVQLSEEFFPVELPEQDQEVED
GQLLQVSGWGYTQNPSESNEALRATNVPAVSQEECRESYGGYQITDRMICAGYQAGGKDACQGDSGGPLVEGKT
LVGVVSWGIGCAEPGYPGVYSREAAVRDWIKEHSGI

> Ochlerotatus epactius (GenBank Acc. No. **AAN75000.1**)

MFASTVIIASFVALSAAYPSIDQGRVVNGQTATLGQFPYQVLLRIQFAEGKALCGGSLLSNQWVLTAGHCTDGAKSF
EVTLGAVDFNSETDDGRVVLTATEYYRHEKYNPLFATNDVAVVKLPQPVEFNDRVHPVELPSGPDSYANQEVVVSG
WGLQKNGGNVADKLQYAPLTVITNDECSQTYSPLVIKKTTLCAKGGNKESPCNGDSGGPLVLEGSKVQVGVVSFG
HAAGCELGYPGAFARVTSFVDWVKKKTGL

Supp. fig. 2.4 Amino acid sequences of early and late trypsins used for alignments and determination of early and late trypsin amino acid motifs.

>HAHN.FLY.9824.C1

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CTTCTAACTGGAATTCTGGTGGCACTTTGGTATCTGTTGCCGCCTTTAAAAATCATGAAGGTTATAACTCTCGTA
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TGGTCCATTGGTTTCCGGTGGTGTTTTGGTTGGTGTTGTCTCCTGGGGCTATGGCTGTGCTTATCCCAATTATCC
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>HAHN.FLY.5249.C1

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>HAHN.FLY.10868.C3

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GCAGCATCCCCTGGCAAATTTCCCTCCAACGTAATGGTGGCCACTCCTGCGGTGGTTCTATCTACAGTTCTAATA
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>HAHN.FLY.124.C2

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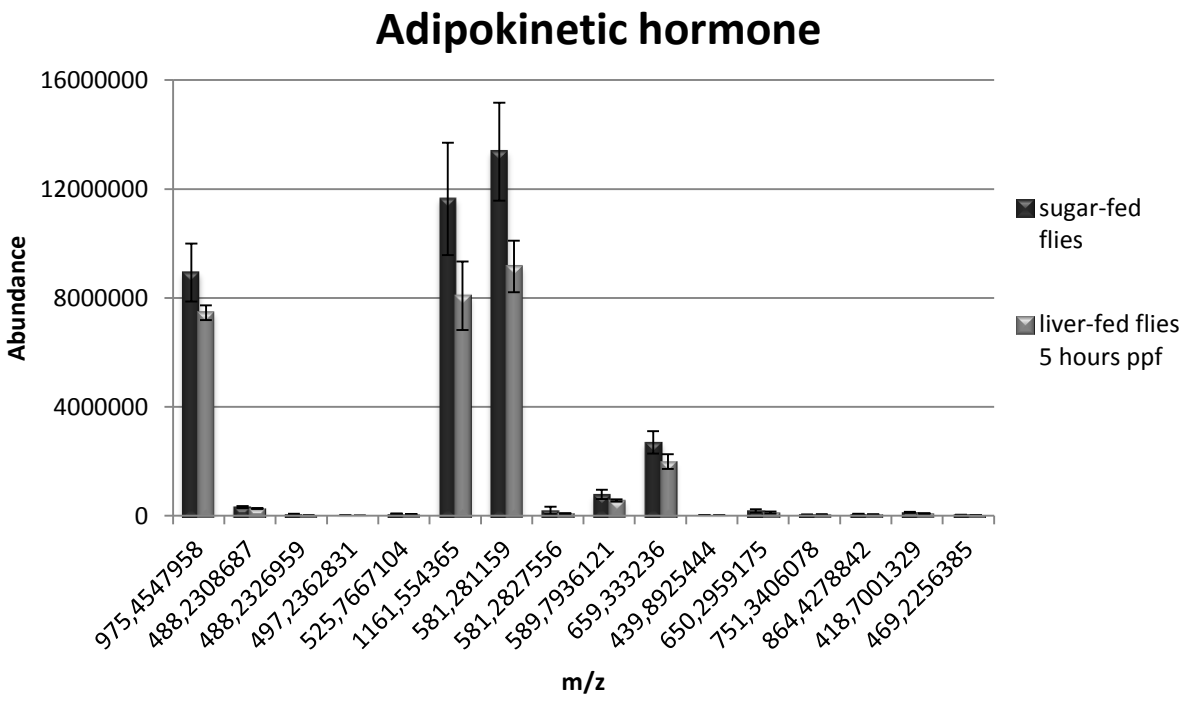
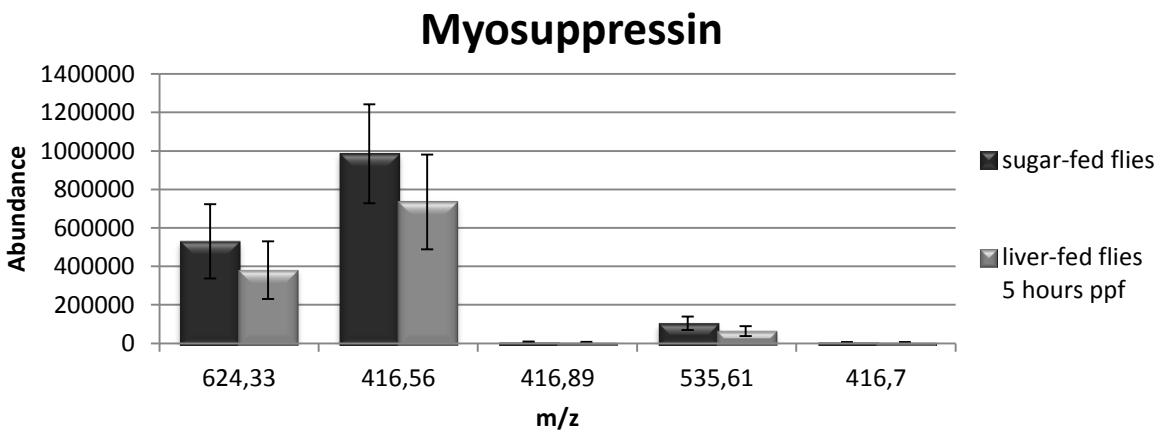
AAATGATTGTGTAAATTTATAAAATGAATTAATAAACGGATCTTTTAAAA

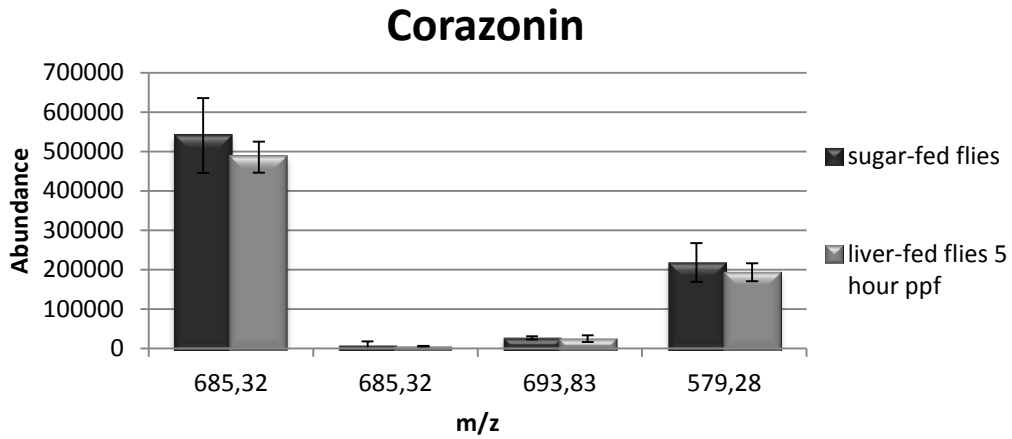
Supp. fig. 2.5 Nucleotide sequences of *S. crassipalpis* trypsin genes. First three sequences are established to be early trypsin genes, whereas the last sequence seems to encode late trypsin.



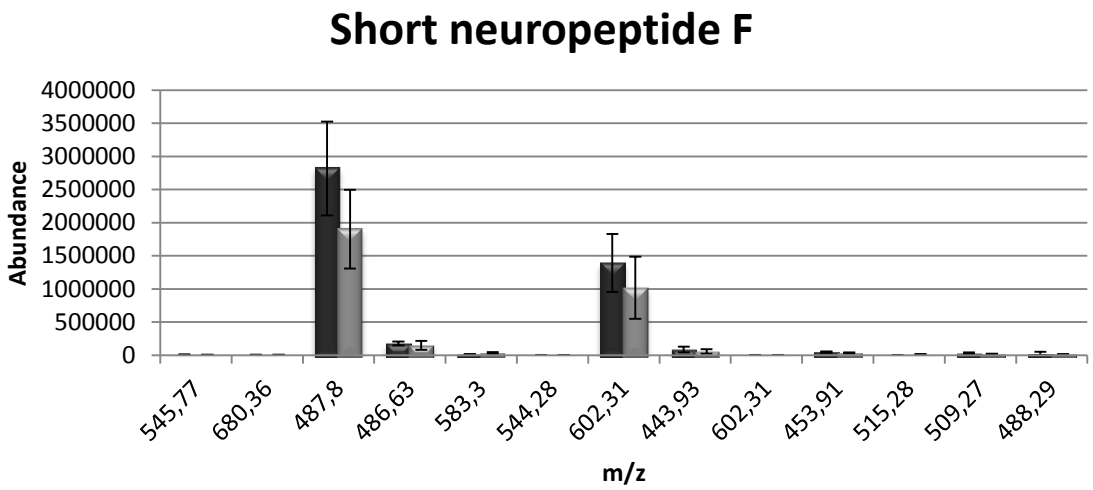
	[M+H]$^+$	m/z	Mass error [ppm]	Charge	Average amount of condition 1	Average amount of condition 2	Sequence	Ratio cond. 2/1	P val
Fully mature peptide									
1	975.45	975.45	2.34	1	8928380	7455855	pQLTFSPDWa	0.84	0.21
2	975.45	488.23	2.68	2	43314.36	24171.04	pQLTFSPDWa	0.56	0.22
3	975.45	488.23	1.07	2	315236.7	261581.7	pQLTFSPDWa	0.83	0.56
Not fully processed peptide intermediates									
4	993.48	497.24	2.40	2	7461.397	11327.95	QLTFSPDW	1.52	0.45
5	1050.54	525.77	0.64	2	64779.63	51724.51	LTFSPDWGK	0.80	0.57
6	1161.55	1161.55	2.68	1	11636519	8078390	pQLTFSPDWGK	0.69	0.17
7	1161.55	581.28	0.65	2	13368794	9154103	pQLTFSPDWGK	0.68	0.50
8	1161.55	581.28	2.10	2	197978.1	77268.87	pQLTFSPDWGK	0.39	0.07
9	1178.58	589.79	3.51	2	778142.1	562519.5	QLTFSPDWGK	0.72	0.43
10	1317.66	659.33	0.39	2	2692335	1990605	pQLTFSPDWGKR	0.74	0.17
11	1317.66	439.89	3.34	3	13374.58	4948.895	pQLTFSPDWGKR	0.37	0.60
Truncated peptide forms									
12	650.30	650.30	4.07	1	175992.1	111939.9	FSPDWa	0.64	0.81
13	751.34	751.34	0.49	1	49202.78	37677.43	TFSPDWa	0.77	0.13
14	864.43	864.43	3.25	1	51633.99	42410.56	LTFSPDWa	0.82	0.76
15	836.40	418.70	0.94	2	125023.6	77035.45	FSPDWGK	0.62	0.75
16	936.46	469.23	2.80	2	31258.77	19926.64	TFSPDWGK	0.64	0.05



	[M+H]$^+$	m/z	Mass error [ppm]	Charge	Average amount of condition 1	Average amount of condition 2	Sequence	Ratio cond. 2/1	P val.
1	1247.66	624.33	0.31	2	529371.7	380322	TDVDHVFLRFa	0.72	0.29
2	1247.66	416.56	0.72	3	985075.7	735358.6	TDVDHVFLRFa	0.75	0.23
3	1248.67	416.89	2.72	3	4867.531	3957.798	TDVDHVFLRF	0.81	0.62
4	1604.83	535.61	4.73	3	104145	63937.48	RKVCQALENSDQLT	0.06	0.11
5	832.4	416.7	2.30	2	4655.007	4614.912	TDVDHVF	0.99	0.96



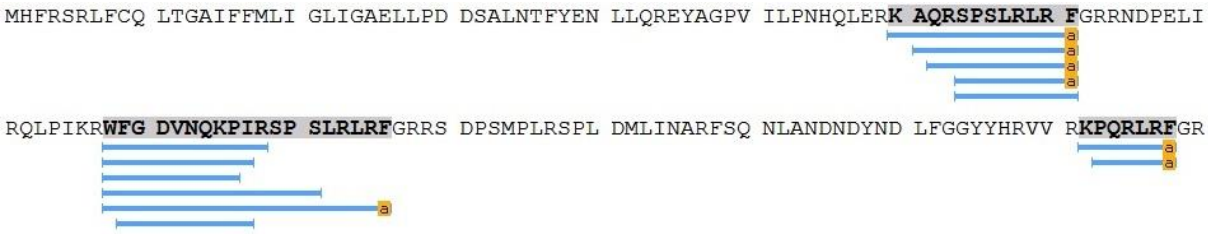
	[M+H]$^+$	m/z	Mass error [ppm]	Charge	Average amount of condition 1	Average amount of condition 2	Sequence	Ratio cond. 2/1	P val.
1	1369.64	685.32	6.38	2	7396.215	4368.73	pQTFQYSRGWTNa	0.59	0.91
2	1369.64	685.32	1.15	2	540412.2	485735.9	pQTFQYSRGWTNa	0.90	0.33
3	1386.66	693.83	0.07	2	26760.88	24704.11	QTFQYSRGWTNa	0.92	0.57
4	1157.56	579.28	1.10	2	217904.9	193241.9	FQYSRGWTNa	0.89	0.44



	[M+H]$^+$	m/z	Mass error [ppm]	Charge	Average amount of condition 1	Average amount of condition 2	Sequence	Ratio cond. 2/1	P val.
1	1090,4	545.77	2.41	2	9481.289	5736.337	WFGDVNQKP	0.60	0.26
2	1359.72	680,36	1.99	2	6371.869	4742.08	WFGDVNQKPIR	0.74	0.27
3	974.62	487,8	1.01	2	2817385	1900240	SPSLRLRF	0.67	0.13
4	973.26	486,63	1.67	3	177547.7	147710.8	KAQRSPSLRLRF	0.83	0.34
5	1165.61	583,3	8.50	2	10646.98	33536.55	QREYAGPVVF	3.15	0.02
6	1087.56	544,28	1.46	3	2434.372	911.8831	WFGDVNQKPIRSPS	0.37	0.26
7	1203.62	602,31	0.48	2	1393122	1016993	WFGDVNQKPI	0.73	0.23
8	886.86	443,93	0.28	3	86253.94	54884.74	AQRSPSLRLRF	0.64	0.24
9	1203.62	602,31	3.09	2	2935.384	2612.791	WFGDVNQKPI	0.89	0.60
10	906.82	453,91	2.61	3	45646.97	34412.16	WFGDVNQKPIR	0.75	0.15
11	1029.54	515,28	9.04	3	2057.98	6536.136	WFGDVNQKPIRSP	3.18	0.76
12	1017.54	509,27	0.83	2	29334.16	14550.6	FGDVNQKPI	0.50	0.07
13	975.58	488,29	2.46	2	24231.57	10077.36	SPSLRLRF	0.42	0.38

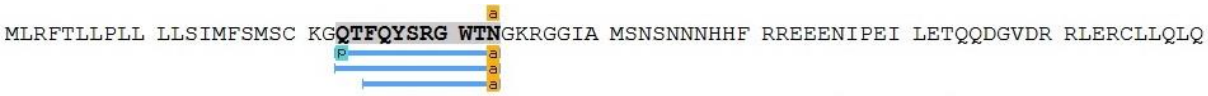
Supp. fig. 3.1 Differential distribution of all detected forms of adipokinetic hormone, myosuppressin, corazonin and short neuropeptide F present in the *corpora cardiaca* of sugar-fed flies (dark blue bars) and liver-fed flies 5 hours ppf (light blue bars)and their numeric values. Condition 1 refers to CC peptidergic extract of sugar-fed flies; whilist condition 2 refers to CC peptidergic content extracted from flies 5 hours ppf. Ratio value between condition 2 and 1 below 1 (observed in almost all cases) indicates decrease/release of the particular peptide in liver-fed flies (4 replicates of 50 pooled CCs each) *± SD.*

Short neuropeptide F



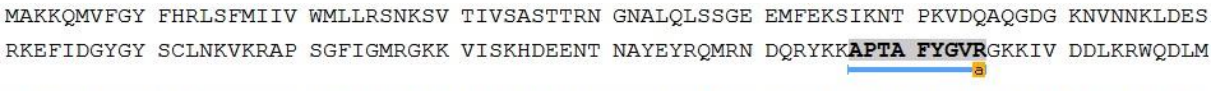
Peptide	Uniq	-10lgP	Mass	ppm	m/z	z	RT	Scan	#Spec	Start	End	PTM
R.WFGDVNQKPI.R	Y	113.19	1202.6084	1.9	602.3126	2	20.64	F6:2409	34	88	97	
R.WFGDVNQKPIR.S	Y	108.82	1358.7095	3.8	453.9122	3	17.62	F6:2033	13	88	98	
R.SPSLRLRF(-.98).G	Y	86.74	973.5821	-1.3	487.7977	2	16.71	F6:1920	41	64	71	Amidation
W.FGDVNQKPI.R	Y	86.71	1016.5291	0.5	509.2721	2	15.92	F2:1817	5	89	97	
K.AQRSPSLRLRF(-.98).G	Y	84.61	1328.7789	-2.2	443.9326	3	14.99	F1:1720	7	61	71	Amidation
R.WFGDVNQKP.I	Y	83.62	1089.5243	1.0	545.7700	2	18.50	F2:2133	4	88	96	
R.WFGDVNQKPIRSPSLRLRF(-.98).G	Y	79.45	2314.2810	-2.8	579.5759	4	22.36	F5:2568	2	88	106	Amidation
R.SPSLRLRF.G	Y	61.61	974.5661	3.2	488.2919	2	18.06	F1:2168	7	64	71	
R.KAQRSPSLRLRF(-.98).G	Y	61.09	1456.8739	1.7	486.6327	3	13.59	F1:1546	10	60	71	Amidation
R.WFGDVNQKPIRSPS.L	Y	51.05	1629.8263	-0.3	544.2825	3	18.00	F4:2108	1	88	101	
R.KPQRLRF(-.98).G	Y	47.75	942.5875	2.5	472.3022	2	10.48	F7:1200	15	152	158	Amidation
A.QRSPSLRLRF(-.98).G	Y	42.38	1257.7418	5.2	420.2567	3	15.06	F4:1714	1	62	71	Amidation
K.PQRLRF(-.98).G	Y	39.51	814.4926	5.7	408.2559	2	10.57	F4:1199	1	153	158	Amidation
total 13 peptides												

Corazonin



Peptide	Uniq	-10lgP	Mass	ppm	m/z	z	RT	Scan	#Spec	Start	End	PTM
G.Q(-17.03)TFQYSRGWTN(-.98).G	Y	110.62	1368.6211	0.4	685.3181	2	21.38	F6:2521	23	23	33	Pyro-glu from Q; Amidation
T.FQYSRGWTN(-.98).G	Y	88.25	1156.5414	-1.4	579.2772	2	16.77	F6:1928	23	25	33	Amidation
G.QTFQYSRGWTN(-.98).G	Y	77.97	1385.6476	-1.1	693.8303	2	21.41	F2:2499	7	23	33	Amidation
total 3 peptides												

Tachykinin



Peptide	Uniq	-10lgP	Mass	ppm	m/z	z	RT	Scan	#Spec	Start	End	PTM
K.APTAFYGVR(-.98).G	Y	111.16	979.5239	-3.7	490.7674	2	16.02	F2:1828	8	137	145	Amidation
total 1 peptides												

CAPA

FQQTKNRRGG SSSSKLLAFP RIGRRDPSMP SNLLNSEAEN TAAVAIDLFY PDLNEEYGEY PKAEYKRASL RAFPRIGRSD
AEIRKWTRLL ALQQALDKR**A GPSATTGVWF GPRL**GKRSTG SDSYE

Peptide	Uniq	-10lgP	Mass	ppm	m/z	z	RT	Scan	#Spec	Start	End	PTM
R.AGPSATTGVWFGPRL(-.98).G	Y	96.81	1514.7993	6.3	758.4117	2	23.36	F8:2696	1	100	114	Amidation
A.GPSATTGVWFGPRL(-.98).G	Y	67.40	1443.7622	2.9	722.8905	2	23.27	F8:2685	1	101	114	Amidation
total 2 peptides												

FMRFamide

MLPLIVYLLA LQHLHTSALS KILLSSDYSL NNFNQDFDKS MRSNDHAVTP TTYEPLLQDI IENVPFVQNF PDATALQFPE
QISSVNIDYG KNVIVLKFSK KPRKLSLNKE EQEKRKSLQE NFMRFGKRAY EYLPTMNDFV GHYYEELPGE RYDRTRETVR
DLR**GDNFMRF** GRNDNQCGNT DCRVTDDFMR FGRSNGGSVD FMRLGRRSGE DFMRFGRNPG NQGFMRFGRS LG**NQDFMRF**G
RNPGNQDFIT FGRNPAEQDF MRSSRNSDFM RFGRSSSSPD FMRFGRNAFD LKMRHEKRSD NFMRFGRASK ENFMRFGKRK
TETKEQLISN QSDLNKKASN ENVTETNPED KSLKTAFDNN QELLNDNDNL MNFEVNNNPL ANDQDALNAD YVSE

Peptide	Uniq	-10lgP	Mass	ppm	m/z	z	RT	Scan	#Spec	Start	End	PTM
R.GDNFMRF(-.98).G	Y	77.12	884.3963	1.1	443.2059	2	18.88	F6:2187	5	164	170	Amidation
R.GDNFM(+15.99)RF(-.98).G	Y	46.96	900.3912	3.1	451.2043	2	16.26	F5:1842	3	164	170	Oxidation (M); Amidation
G.NQDFMRF(-.98).G	Y	38.74	955.4334	0.5	478.7242	2	18.95	F3:2265	1	233	239	Amidation
total 3 peptides												

Supp. fig. 3.2 All detected peptide sequences of the *S. crassipalpis* short neuropeptide F, corazonin, tachykinin, CAPA and FMRFamid situated in the *D. melanogaster* AKH precursors (UniProt Acc. No. **GMOY012142**; **GMOY012060**; **GMOY008567**; **GMOY003803** and **GMOY003383** respectively). Sequence marked in grey is identified as a partial *S. crassipalpis* AKH precursor identical to the *D. melanogaster* sequence. Bleu 'o' and orange 'a' stand for methionine oxidation and amidation.

```
Drome      ---------ATGGCAAAAGTAGCTGAGGAGAATGATCATCGTGATCTAAGTAATTGGTCG
Musdo      ATGTCAGAACAAGAGGTAAATGAGAAAATCTACGACCATCGTGTCCTCACCGACTGGTCG
Glomo      ATGTCCGAAACGGAGGTTAACGGCAAAATCTACGATCATCGTGTTTTACCCCATTGGTCA
                      *        *   *     * ** *******   *     * *****

Drome      AA---TGTGAACGACACCAATGGCACCATTCACCTGACCAAGGATATGGTCTTCAATGAT
Musdo      AATGTCAACAACAACACCAACGGCACCATGCACTACTCGAAGGATATGATTTTCAACGAT
Glomo      AATGTAAAGAACGAGACCAATGGTTCTATTCACTATTCCCCTGACATGATATTCAACGCT
           **       *** * ***** **  * ** ***    *    ** *** * ***** * *

Drome      GGCCACCGATTGTCCATCACCGTGTACAGCATACTGTTTGTGATCTCAACAATTGGCAAC
Musdo      GGCCATCGTTTGTCGATAACAGTCTACAGCATTCTTTTTGTTATCTCAACGATTGGCAAT
Glomo      GGTCATCGGCTATCGATTACAGGTTACAGCATACTGTTTGTACTCTCTCTCATCGGTAAT
           ** ** **  * ** ** ** * *  ******** ** *****  ****    ** ** **

Drome      AGCACCGTGCTGTATCTGCTGACCAAGCGGCGACTGCGC------GGTCCCTTGCGTATT
Musdo      TCAACGGTGTTGTATCTGCTGACCAAGCGACGTTTGCGT------GGTCCATCGCGTATT
Glomo      TCGACGGTATTGTATTTGCTTACTAAGCGGCGTcaTTGAGCAGCCGACATACATCGTATT
              ** **  ***** **** ** ***** **  ** *         * * * *******

Drome      GATATCATGCTAATGCATCTGGCCATCGCCGATCTAATGGTGACGCTGCTCCTGATGCCA
Musdo      GACATCATGCTAATGCATTTGGCAATAGCCGATCTGATGGTGACGTTTCTGCTGATGCCA
Glomo      GATATTATGTTAATGCATCTAGCAATTGCCGATTTAACTGTGACACTATTGTTAATGCCG
           ** ** *** ******** * ** ** ****** * *  *****  *  *  * *****

Drome      ATGGAGATCGTGTGGGCCTGGACGGTGCAGTGGCTATCCACGGACCTGATGTGCCGCCTG
Musdo      TTGGAGATCGCGTGGGCATACACGGTGCAATGGAAATCGACAGACTTCATGTGTCGCCTC
Glomo      TTGGAAGTTGCTTGGTCCTATACGGTGGAATGGAAATCAACCGATTTTATGTGCCGTCTC
            ****  * *  *** * *  ****** * ***  *** ** **  * ***** ** **

Drome      ATGAGCTTTTTCCGCGTCTTTGGCCTGTATCTGTCCAGCTACGTGATGGTCTGCATATCG
Musdo      ATGAGTTTCTTTCGAGTGTTTGGCCTGTATCTATCTGGTTTCGTTTTGGTATGCATATCA
Glomo      ATGAGTTTCTTTAGAGTCTTTGGTTTGTATTTATCAAGTTTCGTCTTGGTATGCATATCA
           ***** ** **  * ** *****  ***** * **  * * ***  **** ********

Drome      CTCGACAGATACTTTGCCATACTGAAGCCGCTCAAGCGGTCCTACAACCGGGGACGCATC
Musdo      ATTGATAGATATTATGCCATATTGAAGCCTCTGAAGCTATCCACTAATCGTGGCCGGATT
Glomo      GTTGACAGATATTTTGCTATAATTAAACCATTAAAAATGTCTACTAACCGTGGGCGACTT
            * ** ***** * *** *** * ** **  * **    **    ** ** ** **  *

Drome      ATGCTGGCCTGCGCCTGGCTGGGATCAGTCGTCTGCAGCATTCCACAGGCCTTTCTCTTC
Musdo      ATGTTGACCATTGCCTGGTGCAGTTCTGTGGTGTGTAGCCTACCTCAGGCCTATTTCTTC
Glomo      ATGTTGTTGGTCGCCTGGTGCACCTCAATTGTATGCAGTTTACCTCAGGCTTTATTATTT
           *** **      ******      **  * ** ** **  * ** ***** *   * **

Drome      CATCTGGAGGAGCATCCCGCCGTCACCGGCTACTTTCAGTGCGTCATCTTCAACTCGTTC
Musdo      CACTTGGAGGAGCATCCCAAAGTGAGCGGCTACTTCCAGTGTGTGACGTTTCACTCATTT
Glomo      CATTTGGGTGAACATCCTAATGTTAAAAACTATTACCAGTGTGTTATGTTTGATGCCTTA
           **  ***  ** *****    ** *    *** *  ***** ** *  **  *  * **

Drome      AGGAGCGACTTTGATGAGAAGCTGTATCAGGCGGCCTCCATGTGCAGCATGTACGCCTTT
Musdo      CCCAGCGAATTCCATCACATTATGTACCAAATTGCCACCATGTGTGCCATGTATGCCTGT
Glomo      ---AGTCAATTTCAATCCATTTTCTACAATATGACAACTATGTGTGCCATGTATGCCTGT
              **  * **  *    *   * **  *     *  * *****   ****** **** *

Drome      CCGCTGATCATGTTCATCTACTGCTACGGAGCCATTTACCTGGAGATCTATCGGAAGAGC
Musdo      CCGTTGATCACCTTTATCTATTGCTACGGTTCCATATATTTGGAAATCTATCGCAAGAGC
Glomo      CCTTTAATCACTTTCATCTATTGTTACGGAGCTATTTATTTGAAAATCTATCGGGAAAGT
           **  * ****  ** ***** ** *****  * ** **  ** * ********  * **
```

```
Drome      CAGCGCGTCCTCAAGGACGTGATTGCCGAGCGTTTCCGGCGTTCCAACGACGACGTGCTC
Musdo      CAGCGCATAGTCAAAGGCA------TTGAACGCTTTCGCCGTTCCAACGATGATGTTTTG
Glomo      AAACGTATGACCAAAGGCG------TGGAACGGTTTCGTCGTTCAAACGATGATGTGCTA
            * **  *   *** * *         ** ** ** ** ***** ***** ** **  *

Drome      AGCCGGGCCAAGAAGCGCACGCTCAAAATGACCATCACGATTGTGATTGTGTTCATCATC
Musdo      AGTCGTGCCAAAAAGCGCACTCTGAAGATGACCATAACCATTGTGATTGTTTTCCTCATC
Glomo      TGTCGAGCCAAGAAGCGCACTTTGAAAATGACTATCACTATTGTCATTGTCTTCATAATA
            * ** ***** ********  * ** ***** ** ** ***** ***** *** * **

Drome      TGCTGGACTCCGTACTACACCATCTCCATGTGGTACTGGCTGGACAAGCACTCCGCGGGC
Musdo      TGCTGGACGCCCTATTACATTATAGCCATGTGGTATTGGTTCGACAAGACATCGGTGGAT
Glomo      TGCTGGACGCCTTATTACATAATTTGCATGTTCTACTGGTTCGATTATAATACAGCTTCA
           ******** ** ** ****  **   *****  ** *** * **  *     * *

Drome      AAGATCAATCCCCTGCTGCGCAAGGCGCTGTTCATCTTCGCCTCGACCAACTCATGTATG
Musdo      AAGGTCAGCGCCCTTGTCAGCAAGTCCCTATTTATATTTGCCTGCACGAATTCATGCATG
Glomo      CGGTTTAGCCCCTTATTGCGTAAAGCATTGTATTTGTTCGCTTGTACTAATTCGTGCATG
             * * *   ** *  *  * **  *  * *   * ** ** *  ** ** ** ** ***

Drome      AATCCATTGGTCTACGGACTCTACAACATTCGCGGGCGAATGAACAACAACAATCCGTCG
Musdo      AATCCCATAGTTTATGGAGCATTCAATATACGTGGACGCATTGGAAATAATGCGTCAACA
Glomo      AATCCCATTGTCTATGGCATGTTTAATATACGAGGACGGAGAGATAACAACGCGAATTCA
           *****  * ** ** **    *  ** ** ** ** ** *     ** **        *

Drome      GTGAACAACAGGCACACCTCGCTGTCCAATCGCCTGGACTCCTCCAATCAGCTGATGCAA
Musdo      ATAAGTAATCGCCATACCTCCCTGTACAATCGTGGAGATTCATCGAATCAATTGCCCAAA
Glomo      TCAAGTAATCGTAATCCGTCCGTGTATCAACGTGGAGATTCATCAAGTCAGATTCCTAAA
              *  **  *  *  * **  ***   * **    ** ** ** * ***  *     **

Drome      AAGCAGTTGACCAACAACTCGCTGCTGAATGGAAGAGGCCAGGTGATGGCAGCCGCGGTT
Musdo      CATTTGCTCAATCTCAGTGAGGGCAGTGCAAAAACGGGCAGTACTCACATCAACCAGACG
Glomo      AGTCTTTTGAATCTGAGCCATAGCGAGAGCAATAAAATACCACCGACAAGGA------CT
                  * *     *                  *

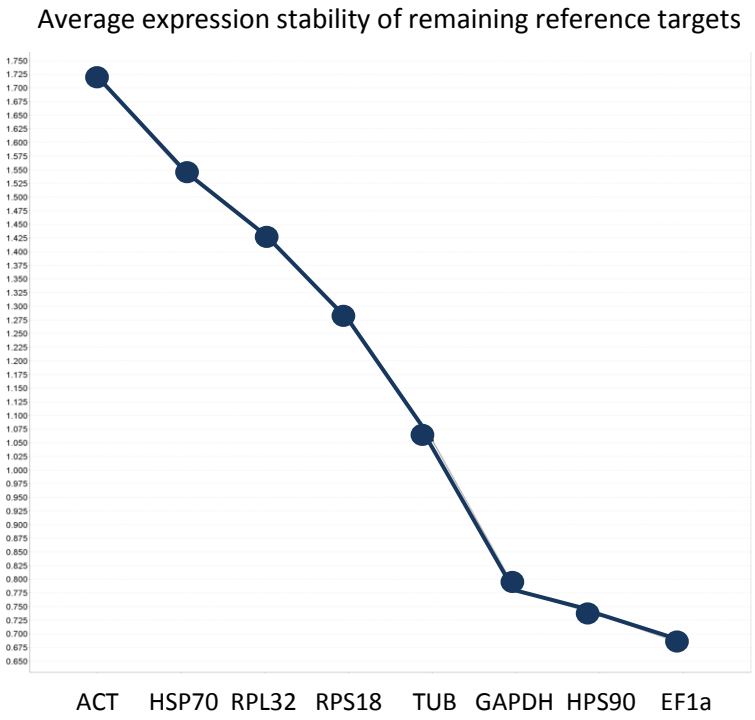
Drome      TCAGCCACCACTAAGTTGGCCAATGTGGTCAGCCTTAAAGGCACTGCCAATGGCAATGGC
Musdo      GACACAACAGATATATCGATCAATCACAAAATCAATGA---------------GACTACA
Glomo      TCGACAAACGATAAATTGGTAGACTCACCGCTGGCCAA----------------------
               * *    **  * *    *              *

Drome      TCAGCTGCAGCCGCGGGAACAGTTCCAATAACTCCTCCGCTGACAGTAACCATCGCTCCG
Musdo      ACAACAGCGAACGGTTGTACAG---AAATCAGTAATAAAGTCGAT-------GAGGTCTC
Glomo      ------GCTAAATAAGGCAGAC---ACATTACCCATGATCTGCAT-------TAGGTAA-
                 **        * * *      ** *    *    *            * *

Drome      CTGGCCACCGACGACGAGGCCAACGATGATTCCTGTCTCAGTGCGGTCACCATCAGGTGC
Musdo      CGGATCACCAGTTAT-----------------ATGTATAAATTGTGTTGACT--------
Glomo      ------------------------------------------------------------


Drome      CAGGATCAATCCCCGATCCGCCAGAAATGTGGCGACTCCATCGAATTGACCAGTGTGGTC
Musdo      CAATTGAACTCTCTAATCGG-CAGAAATCGTAA---------------------------
Glomo      ------------------------------------------------------------
```

Supp. fig. 4.1 Nucleotide sequence alignment of flies' adipokinetic hormone receptors used for primers design assignment. Aligned sequences belong to *Drosophila melanogaster* (Drome), *Musca domestica* (Musdo) and *Glossina morsitans* (Glomo). The first set of primers is marked in blue, the second one in green, and the last one in yellow. The common overlapping parts are marked in grey. Conserved nucleotides of all three sequences are indicated by asterisk.



Supp. fig. 4.2 The geNorm analysis of the housekeeping genes used for qRT-PCR measurement. The average expression stability of all reference genes starting with the least stable genes on the left side and ending with the most stable genes on the right side (top chart). The levels of variations in average reference genes stability with the sequential addition of each housekeeping gene for calculation of the normalization factor of the experiment (bottom chart). Based on the results of these analyses the housekeeping genes selected for this experiment are: EF1α, HSP90 and GAPDH. Abbreviations: EF1α, elongation factor 1α; HSP90, heat shock protein 90; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPS18, ribosomal protein S18; TUB, tubulin; ACT, actin.

>Sarcophaga crasipalpis adipokinetic receptor

MTESEINEKIYDHRVLTDWSNVNNNTNGTMHYSKDMIFNDGHRLSITVYSVLFVISTIGNSTVLYLLTKRR
LRGPSRIDIMLMHLAIADLMVTFLLMPLEIAWAYTVQWKSTDFVCRLMSFFRVFGLYLSSFVLVCISIDRYY
AIIKPLKLSTNRGRIMLAVAWCSSFLCSLPQAYVFHLEEHPKVKGYYQCVTFHSFASEFHKLFYNMANMCA
MYACPLITFIYCYGAIYLEIYRKSQRIVKGIERFRRSNDDVLSRAKKRTLKMTITIVIVFIICWTPYYIICMWYW
IDKSSVDEVSSLIRKSLFIFACTNSCMNPIVYGAFNIRGRINHNNTSTMSNRHTSLYQRGDSSNQLPKHLLN
INGGDSGKIATTNSIVSQENNGNKGTNLNKSEKQKDNDATTQVSMESPPMICISCGDSIEMANLQQKT

>Drosophila melanogaster adipokinetic receptor (GenBank Acc. No. **NP_995639.1**)

MAKVAEENDHRDLSNWSNVNDTNGTIHLTKDMVFNDGHRLSITVYSILFVISTIGNSTVLYLLTKRRLRGP
LRIDIMLMHLAIADLMVTLLLMPMEIVWAWTVQWLSTDLMCRLMSFFRVFGLYLSSYVMVCISLDRYFA
ILKPLKRSYNRGRIMLACAWLGSVVCSIPQAFLFHLEEHPAVTGYFQCVIFNSFRSDFDEKLYQAASMCSM
YAFPLIMFIYCYGAIYLEIYRKSQRVLKDVIAERFRRSNDDVLSRAKKRTLKMTITIVIVFIICWTPYYTISMWY
WLDKHSAGKINPLLRKALFIFASTNSCMNPLVYGLYNIRGRMNNNNPSVNNRHTSLSNRLDSSNQLMQK
QLTNNSLLNGRGQVMAAAVSATTKLANVVSLKGTANGNGSAAAAGTVPITPPLTVTIAPLATDDEANDD
SCLSAVTIRCQDQSPIRQKCGDSIELTSVVK

>Musca domestica adipokinetic receptor (GenBank Acc. No. **XP_005177426.1**)

MSEQEVNEKIYDHRVLTDWSNVNNNTNGTMHYSKDMIFNDGHRLSITVYSILFVISTIGNSTVLYLLTKRR
LRGPSRIDIMLMHLAIADLMVTFLLMPLEIAWAYTVQWKSTDFMCRLMSFFRVFGLYLSGFVLVCISIDRY
YAILKPLKLSTNRGRIMLTIAWCSSVVCSLPQAYFFHLEEHPKVSGYFQCVTFHSFPSEFHHIMYQIATMCA
MYACPLITFIYCYGSIYLEIYRKSQRIVKGIERFRRSNDDVLSRAKKRTLKMTITIVIVFLICWTPYYIIAMWYW
FDKTSVDKVSALVSKSLFIFACTNSCMNPIVYGAFNIRGRIGNNASTISNRHTSLYNRGDSSNQLPKHLLNL
SEGSAKTGSTHINQTDTTDISINHKINETTTTANGCTEISNKVDEVSGSPVICINCVDSIELSNRQKS

>Glossina morsitans adipokinetic receptor (GenBank Acc. No. **AEH25943.1**)

MSETEVNGKIYDHRVLPHWSNVKNETNGSIHYSPDMIFNAGHRLSITGYSILFVLSLIGNSTVLYLLTKRRLS
SRHTSRIDIMLMHLAIADLTVTLLLMPLEVAWSYTVEWKSTDFMCRLMSFFRVFGLYLSSFVLVCISVDRY
FAIIKPLKMSTNRGRLMLLVAWCTSIVCSLPQALLFHLGEHPNVKNYYQCVMFDALSQFQSIFYNMTTMC
AMYACPLITFIYCYGAIYLKIYRESKRMTKGVERFRRSNDDVLCRAKKRTLKMTITIVIVFIICWTPYYIICMFY
WFDYNTASRFSPLLRKALYLFACTNSCMNPIVYGMFNIRGRRDNNANSSSNRNPSVYQRGDSSSQIPKSL
LNLSHSESNKIPPTRTSTNDKLVDSPLAKLNKADTLPMICIR

>Aedes aegypti adipokinetic receptor (GenBank Acc. No. **CAY77166.1**)

MSNAILKTERGEVLNYSHSYGENYNNDVNTMPYVLSSSTSKTGVFDNETWYGTNSSNWNEPLPIDMQF
NDGHKLQIVVYSVLMVISAIGNITVLALLIKRRLKSHSRIDMMLTRLAIADLLVTFLMMPLEIGWAATVQW
RAGDIMCRVMAFFRTFGLHLSSFVLVCISVDRYYAVLQPLNLSKSRGKIMILIAWAMATLCSAPQPFIFHV
EIHPNHTWYEQCVTYNTFSNDNYHTVYNILVMMFMYALPLLTIICSYASIYMEIFRHSRMPNSEGFRRSSI
DALSRAKRRTLKMTITIVMAFVICWAPYYVMSVWYWLDQKSAENVDQRVQKGLFLFACTNSCMNPIVY
GIYNVKLRKKKKPDGVKSGQSSVILRNSAKYTRHSESIRSSSVWF

>Anopheles gambiae adipokinetic receptor (GenBank Acc. No. **ABD60146.1**)

MPNTMAAHINQRIEDHRNLADWSYYANETAGEEYYEMPIDMRFNSGHILSIMVYTTLMVFSATGNLTV
LSILAQRKVRASSRINIMLAHLAIADLLVTFLMMPLEIGWAYTVRWTAGDLMCRVMAFFRTFGLYLSSFILI
CISVDRYFAVLKPLKVHEHRAVLMIAAAWIMSGLCSLPQAFIFHLEGHPNITGYQQCVTYHYFEEEIYQIIY
NVLVMCLMYTFPLIVILYCYGSIYYEIFSRTNPRNLESFRRSSIDVLGRAKRKTLRMTIMIVIVFVVCWTPYYV
MSLWYWLDKESTKNVDQRIQKGLFLFASTNSCMNPVVYGVFNVRKKHTKKLLKTTHEKSCGSHLTMRA

>Apis mellifera adipokinetic receptor (GenBank Acc. No. **NP_001035354.1**)

MESSIKIITTTGLENWRVNNSNYTELLPIDMRFNEGHIVSIVFYSVLMIISAIGNTTVLILITCRKRVSKSRIHI
MLMHLAIADLLVTFLMMPLEIGWAITVSWKAGDVMCRIMAFFRMFGLYLSSFVLVCISMDRYYAVIKPL
QLWDVDKRGKIMLSFAWIGSVVCSLPQTIVFHLETHPNVTWYSQCVTFNAFPTYTHEITYSLFGMIMMY
WFPLVVIIYTYTSILLEIRRRSKKSEDDKIRRSSIGFLTRAKIRTLKMTVIIIAVFFICWTPYYVMSLWYWIDRN
SAYKIDQRIQKGLFLFACTNSCMNPIVYGAFNIRDRNKTSARPTTIETRVTPLSLSLKLLD

>Nasonia vitripennis adipokinetic receptor (GenBank Acc. No. **NP_001161243.1**)

MTTAPVNATTVASLDYDDLPIDMRFNAGHVVSIVTYSILMIISAVGNITVLALLLRRRGNAARTRINTMLIH
LAIADLLVTFLMMPLEIGWAATVSWKAGDAMCRIMSFFRMFGLYLSSFILICISVDRYHAVLRPLQMIDID
RRGRFMIAGSWICSALCSAPQMVVFHVEAHPTFTWYEQCITFNTFPSFTHELTYSLFGMVMMYWFPLIV
IIYTYTSILAEMYRRSKDTTSDRIRRSSLGFLGRARVRTLKMTIIIVLVFFICWTPYYVMSLWYWIDSVTATKV
DLRIQKALFLFACTNSCMNPIVYGAFNIRKGNKVTRNWDIHTLK

>Bomyx mori adipokinetic receptor (GenBank Acc. No. **NP_001037049.1**)

MDIDEKVSGPGGASQKNWSHLLHVNNTYDELPLEMRFNYSHMVSMTVYSVLMVISATGNLTVLYQLVR
RRRAKRASRLDILLMHLAVADLMVTFLMMPLEIAWAGTVQWFAGDLMCRVMMFTRTFGLYLSSFVLIC
IAVDRYYAILKPLNVTWEATVRRAIIVAWVCAGLASLPQSFIFHVEEHPEVKGYNQCVSYGSLPTEKHEFAY
FLVNMILMYVIPLVSTLYCSCAALFEIIRRANTANDKMRRSGIGLLGRARARTLKMTVTIVLVFFTCWSPYY

CYCLWYWIDKESIKNLDPALQKAMWLFSCTNSCANPIVYGVFNRNRWNWRAGKFQNGRCRSGSGRKG
SRLPHGESTEISAATLSRARHSNGSDHNGRRDSSYANQNGPQKHWNTINNNHVTNGMV

>Manduca sexta adipokinetic receptor (GenBank Acc. No. **ACE00761.1**)

MDIEDKVSGPGGASQKNWTHLNTSYDELPLDMRFNHGHMVSMVVYSVLMVVSATGNLTVLSQLVRR
KRAGRASRLDVLLMHLAVADLMVTFLMMPLEIAWAGTVQWLAGDLMCRVMMFTRTFGLYLSSFVLVC
IAIDRYYAILKPLNVTWEARVRRALVVSWVGAGLASLPQSFIFHLEEHPDVKGYFQCVSYGSLPTVHHELA
YFLVNMILMYVIPLVSMLYCSSAALLEIIKRANTSNDKMRRSGVGILGRARARTLKMTVTIVLVFFTCWSPY
YCYCLWYWIDKESIKNLDPAIQKAMWLFSCTNSCANPIVYGLFNRNRWTWRSGHNVRHRSGSMRRGS
RLPYGESMEISAATLNRARNSLGSDRNGRRDSAFNTYNGTQKHWNTINNNHVSNGMV

>Tribolium castaneum adipokinetic receptor (GenBank Acc. No. **NP_001076809.1**)

MNFSETLWKMKDPMSASETVQDHRNLLDWSKTSLDNATEHKLPISMRFNEGHQLSIIVYSILMVFSAIA
NTTVLVLIVKRRRKTPSRINTMLMHLAIADLLVTFLMMPLEIGWASTVSWYAGDAMCRIMMFFRMFGL
YLSSFILVCISVDRFYAVLKPLYLRALDRRDKFMLLGAWLGATLCSIPQMVVFHVESHPNITWYQQCVTYN
VFPTYAHELTYLLFGMVMMYALPLAVIIFSYASILLEIRRRTRNPYGDSVTRSSLAFLGKAKVRTLKMTIIIVL
VFFVCWTPYYVMCIWYWLDRESAKNVDQRIQKALFLFACTNSCMNPVVYGVFNIRARRTGRKVSPRVN
TIKHTSCIPTPNGDSRLPPLEISLKTLE

>Periplaneta americana adipokinetic receptor (GenBank Acc. No. **ABB20590.1**)

MALSSCSDQTLNCTAVITMATPTPSPTVLMEEYMTDDMKFNDGHRMSIITYSILMVVSVAGNSTVLITIL
KRRRTLRYGNNYMFMHLAIADLLVTFLMMPLEIAWNITVSWKGGDLMCRIMLFFRTFGLFLSSFVIVCISL
DRCVAILRPMSKKLLNVARRGKLMLTVAWILATLCSLPQAVIFHVEPHPNVTWYEQCVSFNFFSTKMHEF
TYRVLGMVMMYGLPLIVIVISYACILGEIIRRYQLSPDDSFRRSSLVFLNRARNRTLKMAIIIFVVFFICWTPY
YVMCLWYWIDERSAETVDHRVQKALFLFASTNSCMNPIVYGYFNFRLGRGSGYGATGGRVGQQLHHQ
NVVALSGNSTGLNSRRGSNSSSIYRNNSNQSMSWKNSLRSGRRNSRETEHLHPLPHRNSAQAITNVNGR
DDQHQLHTNSNKTPNVLEDASNSNSKNALTSVICR

>Blatella germanica adipokinetic receptor (Gen Bank Acc. No. **ADL60118.1**)

MTTTELPREQQLTEDMTFGSIHKLCIATYCVLMTVSAIGNITVLVNILKRRRNLRFGNNYMFMHLAIADLL
VTFLMMPLEIGWNATVSWRAGDAACRVMSFFRIFGLYLSSFVIVCISLDRCFAILRPMSNVVNVAKRSRV
MLTTAWSLATVCSLPQVFIFHVQQHPVFTWYEQCLDFDMFPTQLYQFWYRILNMVLVYGFPLLVIFISYA
CILTEIFRRYQLSSDENFRRSSLVFLNRAKNRTLKMAIIIFVVFFICWTPYYVMCLWYWIDQQSAEKVDLRV
RKGLFLFACTNSCMNPIVYGYFNFRSGRGSGYGATRPGQQLQHHQITALSNNSTGVNSRRGSNCSSIYRD
NSNQSMSWNRRSSHETEMHANNNRDENHLHPNSAANHNLRTTVSTVSEVPEAR

>Gryllus bimaculatus adipokinetic receptor (GenBank Acc. No. **ADZ17179.1**)

MEASAAPPMWVLPSNASDGDLQLPRDMTFNSGHVVSIACYSALMVASAAGNLTVLTIILRQRGRARSR
VNHMLMHLAIADLLVTFLMMPLEIAWAATVSWRAGDLLCRLMAVCRVFGLFLSSFVLVCISMDRYFAIL
RPMSLSQVDRRGRIMLTAAWVMSFLCSMPQAMVFSVQSHPTVTWYEQCITWGVLKSHRAEVLYAIFSS
SFMYGIPLLIIIFAYGSIVAEIFRRSRRTGDDVFRRSSLGFLGRAKTRSLKMTLVIVLVFFMCWTPYYIMSVW
YWFDRESAELVDERIRSGLFIFACTNSCMNPIVYGAFNVRRGRGRALREYPESSRCNSQPFSARPRSSAPE
QLQQVAKWRNEKNTQLFQLATNGWDRNGKSSFENIELT

>Rhodnius prolixus adipokinetic receptor (GenBank Acc. No. **AIJ49751.1**)

MTRTEEVFSFTFFPEWSEIRTEKNETYVIPPDMRFNEGHKLALAVYSLLMLISGVGNVWVLVRLAKSRRSR
TNRMLTHLAIADLFVAFLMMPAEILSAATVAWWFGDIPCRIFAFFKTFGLYQSSFVLVCIGIDRYYAIVKPL
SIKDTYCRGKGIVALAWVISGICSLPQVVVFREQEHYTFTGYKQCCTFNAFPTSSHEIAYSMYNMAMMY
MLPLVVIIFCYGSIFIEIYRRTSAQNSGKLRRSTLGFLGRAKNRTLKLTITIILAFFICWTPYYIMALWYWLDRS
TAEGVDVRVKRALFLFACTNSSINPLVYGVYQRTGCGPNNSRTSHNTCITELRQQRTNNHVRENLG

>Homo sapiens gonadotropin-releasing homone(GenBank Acc. No. **NP_000397.1**)

MANSASPEQNQNHCSAINNSIPLMQGNLPTLTLSGKIRVTVTFFLFLLSATFNASFLLKLQKWTQKKEKGK
KLSRMKLLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYLKLFSMYAPAFMMVVISLDRSL
AITRPLALKSNSKVGQSMVGLAWILSSVFAGPQLYIFRMIHLADSSGQTKVFSQCVTHCSFSQWWHQAF
YNFFTFSCLFIIPLFIMLICNAKIIFTLTRVLHQDPHELQLNQSKNNIPRARLKTLKMTVAFATSFTVCWTPYY
VLGIWYWFDPEMLNRLSDPVNHFFFLFAFLNPCFDPLIYGYFSL

>Drosophila melanogaster FMRFamide receptor (GenBank Acc. No. **AAF47700.1**)

MSGTAVARLLLRLELPSPGVMPPPPTDYDYGGPISDDEFLASAMATEGPTVRYDLFPQNNSQPTLQIVLN
HTEVQTDLQYPHYEDLGLDPDPNWTRICEDVYNPLLENNRIEFWVCGVLINIVGVLGILGNIISMIILSRPQ
MRSSINYLLTGLARCDTVLIITSILLFGIPSIYPYTGHFFGYYNYVYPFISPAVFPIGMIAQTASIYMTFTVTLER
YVAVCHPLKARALCTYGRAKIYFIVCVCFSLAYNMPRFWEVLTVTYPEPGKDVILHCVRPSRLRRSETYINIY
IHWCYLIVNYIIPFLTLAILNCLIYRQVKRANRERQRLSRSEKREIGLATMLLCVVIVFFMLNFLPLVLNISEAF
YSTIDHKITKISNLLITINSSVNFLIYIIFGEKFKRIFLLIFFKRRLSRDQPDLIHYESSISNNGDGTLNHRSSGRFS
RHGTQRSTTTTYLVATGGPGGGGCGGGGGNNSLNNVRLTQVSGSPGLVKIKRNRAPSPGPVVYFPARE
MQRSASTTNSTTNNNTSIGYDWTLPDSKKLGHVSSGF

Supp. fig. 4.3 17 amino acid sequences of adipokinetic hormone, gonadotropin-releasing hormone and FMRFamide receptors used for phylogenetic analysis.

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atgacagagtccgagataaatgaaaaaatctacgatcatcgtgttttaaccgattggtca
 M  T  E  S  E  I  N  E  K  I  Y  D  H  R  V  L  T  D  W  S
aatgttaacaacaatacaaatggcacgatgcactattccaaggatatgatatttaacgat
 N  V  N  N  N  T  N  G  T  M  H  Y  S  K  D  M  I  F  N  D
ggtcaccgtttatcgataacagtttatagtgttctctttgtgatctcaacaattggaaac
 G  H  R  L  S  I  T  V  Y  S  V  L  F  V  I  S  T  I  G  N
tcaaccgtgttgtatctattaacgaagcgacgattacgtggtccgtcgcggattgacatc
 S  T  V  L  Y  L  L  T  K  R  R  L  R  G  P  S  R  I  D  I
atgttaatgcatttggcgattgccgatctaatggtgacgttcctgctgatgccgctagag
 M  L  M  H  L  A  I  A  D  L  M  V  T  F  L  L  M  P  L  E
attgcgtgggcctatacggtgcaatggaaatcgacagatttcgtttgtcgtctcatgagt
 I  A  W  A  Y  T  V  Q  W  K  S  T  D  F  V  C  R  L  M  S
ttctttagagtatttggtctctacctatcgagtttcgtgctggtatgcatatccattgac
 F  F  R  V  F  G  L  Y  L  S  S  F  V  L  V  C  I  S  I  D
agatattatgccataattaaacctttgaaactttctacaaatcgtggccgcatcatgttg
 R  Y  Y  A  I  I  K  P  L  K  L  S  T  N  R  G  R  I  M  L
gccgttgcctggtgtagttccttttatgtagtctgccacaggcctatgtattccacttg
 A  V  A  W  C  S  S  F  L  C  S  L  P  Q  A  Y  V  F  H  L
gaggagcatccaaaagttaaaggctattatcagtgtgttacatttcattcatttgccagt
 E  E  H  P  K  V  K  G  Y  Y  Q  C  V  T  F  H  S  F  A  S
gaatttcacaaattgttttataatatggccaacatgtgtgccatgtatgcctgtccattg
 E  F  H  K  L  F  Y  N  M  A  N  M  C  A  M  Y  A  C  P  L
atcacctttatctattgctacggggcgatctatttggaaatttatcgcaagagccagcgc
 I  T  F  I  Y  C  Y  G  A  I  Y  L  E  I  Y  R  K  S  Q  R
atcgttaaaggcattgaacgatttcgccgttccaatgatgatgttttaagtcgtgcaaag
 I  V  K  G  I  E  R  F  R  R  S  N  D  D  V  L  S  R  A  K
aagagaacattaaaaatgactataaccattgttattgtctttataatatgttggacgccc
 K  R  T  L  K  M  T  I  T  I  V  I  V  F  I  I  C  W  T  P
tactatattatctgtatgtggtattggatcgataaatcctcagtggatgaggtcagttcc
 Y  Y  I  I  C  M  W  Y  W  I  D  K  S  S  V  D  E  V  S  S
ttaatacgcaagagcctattcatatttgcttgcactaactcgtgcatgaatcccattgtc
 L  I  R  K  S  L  F  I  F  A  C  T  N  S  C  M  N  P  I  V
tatggagcgtttaatatacgtggacgtataaatcataacaacacgtccactatgagtaat
 Y  G  A  F  N  I  R  G  R  I  N  H  N  N  T  S  T  M  S  N
cgacatacgtctttgtatcaacgtggcgattcatccaatcaattgccaaaacatttgtta
 R  H  T  S  L  Y  Q  R  G  D  S  S  N  Q  L  P  K  H  L  L
aatataaatggtggcgacagtggcaaaatagcaacaacaaactccatagtatcgcaagaa
 N  I  N  G  G  D  S  G  K  I  A  T  T  N  S  I  V  S  Q  E
aataatggcaacaaggggacgaatttaaataaaagtgaaaaacaaaaggacaatgatgct
 N  N  G  N  K  G  T  N  L  N  K  S  E  K  Q  K  D  N  D  A
acaacacaagtatcaatggaatcaccacccatgatttgcataagttgcggggactctatt
 T  T  Q  V  S  M  E  S  P  P  M  I  C  I  S  C  G  D  S  I
                gaaatggctaatctacaacaaaaaacataa
                 E  M  A  N  L  Q  Q  K  T  -
```

Supp. fig. 4.4 Nucleotide and corresponding amino acid sequence of *Sarcophaga crassipalpis* adipokinetic hormone receptor.

List of publications

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